UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Biotecnologia

Tese

BioBricks: desenho racional de vacinas de *Mycobacterium bovis* BCG recombinante

Thaís Larré Oliveira

Pelotas, 2018

Pelotas, 2018

Thaís Larré Oliveira

BioBricks: desenho racional de vacinas de *Mycobacterium bovis* BCG recombinante

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutora em Ciências (áreasdo Conhecimento: Biologia Molecular e Imunologia).

Orientador: Odir Antônio Dellagostin

Coorientadora: Caroline Rizzi

Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogação na Publicação

O48b Oliveira, Thaís Larré

Biobricks : desenho racional de vacinas de Mycobacterium bovis BCG recombinante / Thaís Larré Oliveira ; Odir Antônio Dellagostin, Caroline Rizzi, orientadores. — Pelotas, 2018.

83 f.

Tese (Doutorado) — Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, 2018.

1. Biobricks. 2. BCG recombinante. 3. Vacinas. 4. Leptospirose. I. Dellagostin, Odir Antônio, orient. II. Rizzi, Caroline, orient. III. Título.

CDD : 614.56

Elaborada por Ubirajara Buddin Cruz CRB: 10/901

BANCA EXAMINADORA

Profª. Drª. Sibele Borsuk (CDTec, UFPel)

Profª. Drª. Fabiana Kömmling Seixas (CDTec, UFPel)

Dr. Marco Alberto Medeiros (Bio-Manguinhos, Fundação Oswaldo Cruz)

Prof. Dr. Odir Antônio Dellagostin (Orientador - CDTec, UFPel)

Aos meus pais, Cristina e Valter, e à minha irmã Júlia,por todo amor, apoio e suporte durante esta trajetória.
Com amor, dedico.

Agradecimentos

À Universidade Federal de Pelotas, ao Núcleo de Biotecnologia do Centro de Desenvolvimento Tecnológico e à CAPES pela oportunidade de realizar o doutorado em um programa de excelência e pela concessão da bolsa;

Ao professor Odir Antônio Dellagostin, pelo exemplo, pela orientação desde a iniciação científica, pelas oportunidades de crescimento e por toda confiança depositada em mim e no meu trabalho;

À minha coorientadora Caroline Rizzi, por todo o aprendizado neste período, pelo apoio, pela disposição em ensinar e pela fundamental ajuda nesse trabalho;

À professora Daiane Hartwig, pelos ensinamentos, confiança, apoio, oportunidades e compreensão em toda minha trajetória acadêmica;

Aos colegas Carlos Eduardo Cunha, Amilton Seixas e Jessica Dornelles, por toda ajuda e pela essencial contribuição nesse trabalho;

A toda equipe do Biotério Central, fundamental para realização deste trabalho, em especial à Fabiane Carvalho pela dedicação e amizade;

Aos demais professores, amigos e colegas da Biotecnologia, pelos momentos de descontração e por tornarem esta caminhada mais leve, especialmente à Karen Leal, Michele dos Santos, Sérgio Jorge, Violetta Pacce, Natasha Oliveira, Caroline Lucas, Mariana Remião, Marcelle Silveira e Neida Conrad;

À minha família, pelos valores, pelo amor e pelo apoio e suporte em toda minha formação.

Muito obrigada!

Resumo

OLIVEIRA, Thaís. **BioBricks: desenho racional de vacinas de *Mycobacterium bovis* BCG recombinante.** 2018. 83f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Desde a década de 90, *Mycobacterium bovis* BCG tem sido geneticamente manipulado para utilização como vetor vacinal. Entretanto, a falta de padronização na clonagem de sequências de DNA para a construção de BCG recombinante (rBCG) resulta em protocolos não reprodutíveis e eficácia variada. A estratégia BioBricks permite padronizar a construção de moléculas de DNA pelo uso de sequências prefixo e sufixo que flanqueiam os fragmentos alvo. Nesta tese, a tecnologia BioBricks foi aplicada para desenvolver um kit, chamado de caixa de ferramentas, contendo sequências de DNA de micobactéria importantes para a construção de cepas de rBCG. Além disso, vetores epissomais compatíveis com o padrão BioBricks, capazes de se replicar em BCG, foram desenvolvidos e caracterizados através de microscopia de fluorescência e citometria de fluxo, utilizando a proteína repórter eGFP. Estes vetores foram aplicados para a expressão de proteínas quiméricas de *Leptospira interrogans* em rBCG, as quais foram avaliadas como vacinas vetorizadas contra leptospirose em hamsters. O *kit* construído para micobactéria, utilizando o padrão BioBricks, contém dez promotores, duas sequências codificadoras, um plasmídeo base, um gene repórter, um gene de resistência à canamicina e a origem de replicação em micobactéria. Todas estas sequências de DNA são funcionais e compatíveis entre si. Além disso, a funcionalidade de sete vetores epissomais construídos utilizando diferentes promotores foi demonstrada pela detecção da expressão de eGFP em rBCG cultivado *in vitro* e no interior de macrófagos. Estes vetores estão disponíveis para expressão de quaisquer antígenos heterólogos em BCG, clonados com o padrão BioBricks. As vacinas vetorizadas por BCG usando os plasmídeos construídos neste estudo foram capazes de proteger hamsters de desafio letal com *L. interrogans* e do estado de portador renal desta espiroqueta. Juntos, estes resultados representam um avanço para a construção de cepas de rBCG, trazendo praticidade para este processo, e salientam o potencial de BCG como um veículo vacinal contra leptospirose.

Palavras-chave: BioBricks, BCG recombinante, vacinas, leptospirose

Abstract

OLIVEIRA, Thaís. **BioBricks: rational design of recombinant *Mycobacterium bovis* BCG vaccines.** 2018. 83f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Since the 90s, *Mycobacterium bovis* BCG has been genetically engineered for use as a vaccine vehicle. However, the lack of standardization in cloning sequences from mycobacteria to construct rBCG results in poorly reproducible protocols and unpredictable efficacy. BioBricks strategy allows to standardize the construction of basic parts of DNA by the use of prefix and suffix tags flanking the target sequence. In this thesis, the BioBrick technology was applied to develop a kit, named a toolbox, containing mycobacterial DNA sequences, important for construction of rBCG strains. Additionally, BioBrick-compatible episomal vectors able to replicate in BCG were developed and characterized by fluorescence microscopy and flow cytometry using the reporter protein eGFP. These vectors were applied for the expression of chimeric proteins from *Leptospira interrogans* in rBCG, which were evaluated as vectorized vaccines against leptospirosis in hamsters. The kit constructed for mycobacteria according to the BioBricks standard contains ten promoters, two coding sequences, a base plasmid, a reporter gene, a kanamycin resistance gene and the origin of replication in mycobacteria. All these DNA sequences are functional and compatible with each other. In addition, the functionality of seven episomal vectors constructed using different promoters was demonstrated by detecting eGFP expression in BCG grown in vitro and inside macrophages. These vectors are available for expression of any heterologous antigens in BCG, cloned with the BioBricks standard. Vaccines vectorized by BCG using the plasmids constructed in this study were able to protect hamsters against lethal challenge with *L. interrogans* and from the renal carrier status of this spirochete. Taken together, these findings represent substantial progress for construction of rBCG strains, bringing facility for this process, as well as highlight the potential of BCG as a vaccine vehicle against leptospirosis.

Keywords: BioBricks, recombinant BCG, vaccines, leptospirosis.

Lista de Abreviaturas

BCG – Bacilo Calmette-Guérin

rBCG – BCG recombinante

EMJH – Ellinghausen-McCullough-Johnson-Harris *medium*

PBS – Tampão Salina de Fosfato (*Phosphate Buffer Saline*)

SDS-PAGE – Eletroforese em gel de acrilaminda – dodecil sulfato de sódio

CONCEA – Conselho Nacional de Controle da Experimentação Animal

BSA – Albumina sérica bovina (Bovine Serum Albumin)

CDS – Sequencia codificadora (Coding)

LD50/DL50 – Dose letal à 50% dos animais

LPS – Lipopolissacarídeo

OMP – Proteínas de membrana externa (Outer Membrane Protein)

MIT – Massachusetts Institute of Technology

eGFP – Proteína verde fluorescente mutada (Enhanced Green Fluorescent Protein)

TB – Tuberculose

CDS – Sequência codificadora de DNA (Coding DNA Sequence)

ELISA – Ensaio imunoenzimático (Enzyme-linked Immunosorbent Assay)

CFU – Unidades formadoras de colônia (Colony Forming Units)

PCR – Reação em Cadeia da Polimerase (Polymerase Chain Reaction)

qPCR – PCR quantitativo em tempo real

Q1 – Quimera 1

Q2 – Quimera 2

Sumário

1 INTRODUÇÃO GERAL

Mycobacterium bovis BCG é uma vacina mundialmente empregada contra tuberculose, a qual oferece grande potencial para uso como vetor vacinal contra outros patógenos (Singh et al., 2016). BCG apresenta uma série de vantagens: pode ser administrada ao nascimento; replica-se em células apresentadoras de antígenos como macrófagos e células dendríticas, sendo, portanto, eficaz na apresentação de antígenos ao sistema imune do hospedeiro e capaz de conferir proteção de longa duração, induzindo imunidade Th1 e Th2; é estável e não requer refrigeração (Ohara & Yamada, 2001). Além disso, a parede celular de micobactéria é um dos mais fortes adjuvantes imunológicos conhecidos (adjuvante de Freund completo), uma característica que também é explorada na imunoterapia de carcinoma de bexiga (Lee et al., 2004, Quan et al., 2017).

Desde seu desenvolvimento inicial na década de 90, as vacinas de BCG recombinante (rBCG) têm sido desenvolvidas para expressar antígenos homólogos e heterólogos de uma variedade de microrganismos (Bastos et al., 2009, Matsuo & Yasutomi, 2011). A expressão de genes heterólogos é alcançada pelo controle de uma série de promotores micobacterianos. O direcionamento dos antígenos alvo para o meio extracelular ou para a superfície do BCG - permitindo sua associação com componentes adjuvantes da parede celular e sua consequente exposição ao sistema imune – é possível pela inclusão de sequências sinal de micobactéria (Matsuo et al., 1990, Kaufmann & Hess, 1999, Bastos et al., 2009, Oliveira et al., 2017). Até o momento, a expressão heteróloga em rBCG tem sido dirigida por um número relativamente pequeno de promotores, enquanto que a força e atividade regulatória *in vitro* e *in vivo* desses promotores também não foram bem caracterizadas. A atividade de diferentes promotores pode alterar os níveis de expressão do antígeno e a estabilidade do vetor vacinal, o que pode alterar a resposta imune induzida pelas vacinas de rBCG (Oliveira et al., 2017).

BioBricks é uma tecnologia estabelecida em 2003 por Tom Knight para padronizar a construção de sistemas biológicos e a clonagem de sequências de DNA, sejam elas promotores, sequências codificadoras, esqueletos plasmidiais, entre outras ‘partes’ (Shetty et al., 2008). Todos os elementos construídos como BioBricks são depositados em um repositório de partes criado em um estudo do Massachusetts Institute of Technology (MIT) (Smolke, 2009). Esse repositório funciona como um banco de partes padronizadas e compatíveis, as quais são desenhadas e depositadas em um mesmo plasmídeo base. Já foram depositadas mais de 2 mil partes básicas, algumas destas organizadas em coleções, que abrigam sequências relacionadas, como sequências de *Escherichia coli* ou *Bacillus subtilis*, por exemplo(Levskaya et al., 2005, Constante et al., 2011, Radeck et al., 2013, Sureka et al., 2014, Popp et al., 2017).

A estratégia BioBricks consiste em um protocolo simples: sequências prefixo (que possuem sítios para as enzimas de restrição *Eco*RI e *Xba*I) e sequências sufixo (sítios *Spe*I e *Pst*I) são projetadas para flanquear as regiões terminais 5' e 3' de uma porção genética, respectivamente (Casini et al., 2015). Estas sequências prefixo e sufixo são idênticas para todas as partes, tornando-as compatíveis entre si. Durante a montagem de quaisquer duas peças BioBricks, a extremidade *upstream* 3' de um dos fragmentos de DNA a ser montado é digerido com *Spe*I e a extremidade *downstream* 5' do outro fragmento é digerida com *Xba*I. Já que *Spe*I e *Xba*I produzem extremidades coesivas compatíveis, torna-se possível a ligação de uma parte à outra de forma adjacente, formando uma nova construção que conserva todos os sítios originais do prefixo e sufixo e, portanto, segue sendo um elemento BioBrick compatível com todas as outras partes . Assim, esta nova ‘peça’ pode ser novamente digerida para etapas subsequentes de montagem em um processo prático, padronizado e reprodutível com etapas de digestão e ligação consecutivas e idênticas, permitindo a montagem de inúmeras combinações (Muller & Arndt, 2012).

Assim, o objetivo deste trabalho foi desenvolver, caracterizar e aplicar o sistema BioBricks ao BCG recombinante. O documento está dividido em 3 capítulos: o primeiro apresenta um artigo de revisão sobre a influência de características moleculares nas vacinas de rBCG publicado no periódico *Applied Microbiology and Biotechnology*; o segundo apresenta o artigo científico que descreve o desenvolvimento e a caracterização de uma “caixa de ferramentas” composta por sequências de DNA compatíveis com a tecnologia BioBricks e importantes para o trabalho e para a construção de rBCG; por fim, o terceiro capítulo apresenta o artigo em que essa caixa de ferramentas foi aplicada para a construção de cepas de rBCG expressando proteínas quiméricas de *Leptospira interrogans*, as quais foram avaliadas como vacinas vetorizadas contra leptospirose em hamsters a fim de demonstrar seu potencial imunoprotetor e a aplicação do sistema BioBricks desenvolvido para expressão de antígenos de outros patógenos. A tese também possui em anexo dois pedidos de registro de patente: o primeiro referente às construções obtidas para o trabalho com rBCG utilizando o método BioBricks e o segundo referente à utilização de proteínas quiméricas como antígenos vacinais contra leptospirose.

2 HIPÓTESE E OBJETIVOS

2.1 Hipótese

A utilização da tecnologia BioBricks aplicada ao BCG disponibiliza as ferramentas necessárias para o desenho racional de vacinas vetorizadas por BCG recombinante.

2.2 Objetivo Geral

Desenvolver, caracterizar e aplicar a tecnologia BioBricks para *Mycobacterium bovis* BCG, bem como demonstrar a funcionalidade desse sistema para expressão de antígenos de *Leptospira interrogans* em BCG recombinante.

2.3 Objetivos Específicos

- Construir sequências de DNA de *Mycobacterium bovis* BCG compatíveis com o padrão BioBricks®;
- Construir vetores de expressão em BCG compatíveis com o padrão BioBricks® contendo os seguintes promotores micobacterianos: pAN, 18 kDa, Hsp60, HspX e HspX truncado, e Ag85B com ou sem peptídeo sinal;
- Obter cepas de rBCG expressando eGFP como proteína repórter sob o controle de diferentes promotores micobacterianos;
- Avaliar a atividade dos promotores usando eGFP como proteína repórter em BCG cultivado *in vitro* e dentro de macrófagos;
- Construir cepas de rBCG expressando quimeras compostas por frações dos genes *lipL32*, *lemA*, *ligA* e *ligBrep* de *Leptospira* spp. patogênicas utilizando o padrão BioBricks®;
- Imunizar hamsters sírios (*Mesocricetus auratus*) com as cepas de rBCG expressando as proteínas quiméricas compostas por antígenos de *Leptospira* spp. para avaliar o potencial protetor e esterilizante destas vacinas contra leptospirose, bem como a resposta imune por elas induzida;

3 CAPÍTULOS

3.1 Artigo 1

Recombinant BCG vaccines: molecular features and their influence in the expression of foreign genes

Artigo publicado no periódico *Applied Microbiology and Biotechnology*

DOI 10.1007/s00253-017-8439-6

MINI-REVIEW

Recombinant BCG vaccines: molecular features and their influence in the expression of foreign genes

Thaís Larré Oliveira[1] & Caroline Rizzi[1] & Odir Antônio Dellagostin[1,2]

Received: 3 April 2017 / Revised: 13 July 2017 / Accepted: 15 July 2017 / Published online: 4 August 2017

Abstract Recombinant *Mycobacterium bovis* BCG vaccines (rBCG) were first developed in the 1990s as a means of expressing antigens from multiple pathogens. This review examines the key structural factors of recombinant *M. bovis* that influence the expression of the heterologous antigens and the generation of genetic and functional stability in rBCG, which are crucial for inducing strong and lasting immune responses. The fundamental aim of this paper is to provide an overview of factors that affect the expression of recombinant proteins in BCG and the generation of the immune response against the target antigens, including mycobacterial promoters, location of foreign antigens, and stability of the vectors. The reporter systems that have been employed for evaluation of these molecular features in BCG are also reviewed here.

Keywords Recombinant BCG · Vaccine · Antigen expression · Molecular features · Stability · Reporter systems

Introduction

Mycobacterium bovis Bacillus Calmette-Guerin (BCG) is a strain of *M. bovis* that was attenuated in vitro over a 13-year period. Since 1928, BCG has been widely used as a vaccine against human tuberculosis. The recombinant BCG, which expresses immunogenic antigens or modulates the immune response, represents a promising strategy through which live recombinant vaccines can be developed (Singh et al. 2016). BCG offers a number of unique and beneficial features that makes it suitable as a vaccine vehicle: it is inexpensive to produce, is stable and safe, presents inherent adjuvant properties, is uninfluenced by maternal antibodies, can be administered orally, and is highly immunogenic, and it replicates inside macrophages, which means that it can be administered as a single dose to elicit a long-lasting immunity (Bastos et al. 2009).

The expression of foreign antigens from bacteria, parasites, and viruses has been reported using BCG as a vector, and researchers have found that it has the ability to induce both humoral and cellular immune responses in animal models (Bastos et al. 2009). Another strategy for modulating the host immune response using rBCG is to engineer strains to express cytokines, such as interleukin-2 and gamma-interferon (Kong and Kunimoto 1995; Murray et al. 1996). Similarly, the expression of cytolysins represents an alternative means by which BCG can be facilitated to escape from phagolysosome, promoting antigen presentation via MHC I and resulting in CD8 T cell activation (Grode et al. 2005; Sun et al. 2009).

Research efforts have focused on improving the efficacy of BCG, which fails to protect adults against tuberculosis (TB), especially in countries where TB is endemic. Several studies have focused on the overexpression of immunodominant antigens of BCG and on the reintroduction of any beneficial antigens that may have been lost during the BCG attenuation process (da Costa et al. 2014b). The main antigens that have been evaluated in research efforts thus far have been HspX protein and those from Ag85B complex (Liang et al. 2015; Yuan et al. 2015), while the reintroduction of potentially deleted antigens in BCG has focused mainly on ESAT-6 and CFP-10 antigens (Cockle et al. 2002; da Costa et al. 2014b).

* Odir Antônio Dellagostin
odirad@terra.com.br

[1] Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, RS, Brazil

[2] Unidade de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário, Caixa Postal 354, Pelotas, RS CEP 96010-900, Brazil

Delivery of the heterologous or native genes has usually been via a plasmid or integrative vector, and the expression of the foreign genes occurs by the control of a range of mycobacterial promoters (Bastos et al. 2009). However, certain aspects related to the metabolic load, toxicity, and location of the heterologous antigen, and the stability of the vectors in vivo, need to be better exploited to enhance the predictability of rBCG in terms of its efficacy. This review describes a number of molecular factors that are considered to be critical for the expression of recombinant proteins in BCG, and the generation of the immune response against the target antigens.

Mycobacterial promoters

The expression of a target gene is initially regulated in transcription and partially determined by the strength of the promoter. The selection of an effective promoter is crucial to achieving an optimal transcription level and optimizing protein production (Newton-Foot and Gey van Pittius 2013). A useful characteristic of an rBCG-active promoter would be expressed at low levels in vitro thereby maximizing stability and avoiding a high metabolic load. However, high levels are required during the intracellular growth of rBCG in the antigen-presenting cells, inducing antigen expression when an immune response is necessary (Dellagostin et al. 1995). The inducible expression systems may represent an attractive alternative by which the toxicity and metabolic burden can be reduced during in vitro growth (Schnappinger and Ehrt 2014). The regulation of classic, alternative, inducible, and engineered promoters, and the consequences these promoters have on protein production, is reviewed below.

Classic promoters

Hsp60 The Hsp60 is a constitutive and strong mycobacterial promoter that is extensively used for the expression of foreign antigens in BCG. Some studies have reported the induction of Hsp60 promoter in stress conditions related to temperature shifts and during intracellular growth (Batoni et al. 1998; Stover et al. 1991). The Hsp60 promoter drives the expression of antigens in high levels both in vitro and in vivo (Medeiros et al. 2002). Such levels may be desirable for the induction of a strong immunity and the expression and monitoring of reporter genes (Singh et al. 2014; Zhang et al. 2012). However, high-level expression in vitro may be detrimental for the expression of toxic proteins for mycobacteria. Moreover, structural instability has been associated with the use of this promoter, resulting from deletions in its sequence (Al-Zarouni and Dale 2002). In one study, rBCG expressing an antigen from *Leptospira interrogans* under the control of the Hsp60 promoter failed to induce immunity in an animal model, and this failure was attributed to the instability of the promoter (Seixas et al. 2007).

Hsp70 The Hsp70 promoter is conserved and upregulated in response to heat and hydrogen peroxide stress (Cheng et al. 1997). It presents functional activity in *M. bovis* BCG, *M. smegmatis*, and *M.vaccae* to similar and moderated levels (Medeiros et al. 2002). The Hsp70 protein is highly conserved and expressed within macrophages (Batra. 2012). These characteristics entail that the promoter has significant potential as a means of driving the expression of heterologous antigens in BCG.

Alternative promoters

18 kDa The 18 kDa protein is a major antigen during *M. leprae* natural infection. The *M. leprae* 18 kDa promoter exhibits low levels of expression in bacterial culture but shows high levels of expression in BCG cells infecting macrophages (Dellagostin et al. 1995). Indeed, its induction was shown during nutrient depletion, low oxygen tension, and oxidative stress conditions (Maheshwari and Dharmalingam 2013). These studies demonstrated the activation of 18 kDa promoter during intracellular growth, which is a desirable characteristic for the expression of vaccine candidates in rBCG.

pAN The pAN promoter from *M. paratuberculosis* presents homologous sequences in the major pathogenic groups of mycobacteria (Gormley et al. 1997). It is able to drive expression of foreign antigens in both *M. smegmatis* and *M. bovis* BCG (Medeiros et al. 2002). The expression of β-galactosidase under the control of the pAN promoter in BCG has been found to elicit humoral and cellular immunity in mice (Murray et al. 1992). In two studies, a comparison of vectors that contained different promoters revealed that the stability of the BCG was superior when the pAN promoter was used (Medeiros et al. 2002; Seixas et al. 2007).

mtrAThe MtrA protein is part of a conserved system of signal transduction in mycobacteria (Bretl et al. 2011). Essential for mycobacteria growth, it presents differential activity in virulent strains of *M. tuberculosis* and in BCG (Zahrt and Deretic 2000), where expression is upregulated inside macrophages (Via et al. 1996). In one study, the L1 antigen from cottontail rabbit papillomavirus was expressed in BCG using the mtrA promoter and was found to induce low levels of neutralizing antibodies in rabbits, suggesting that the level of antigen expression under the control of this promoter was low (Govan et al. 2006). Chapman et al. demonstrated a stable pantothenate auxotroph BCG strain that expressed the Gag protein under the control of the mtrA promoter (Chapman et al. 2012).

pBlaF The pBlaF promoter drives the expression of the β-lactamase gene from the *M. fortuitum* (Timm et al. 1994b). The pBlaF promoter has been found to produce higher levels of heterologous antigens than the pAN promoter (Abdelhak et al. 1995; Lagranderie et al. 1997; Timm et al. 1994a), although no attempt has been made to evaluate the influence the plasmid copy number has on the expression levels obtained. The expression levels of *Anaplasma marginale* MSP1a antigen in rBCG have been found to be lower when the pBlaF promoter is used than when the 18 kDA promoter is applied. Nevertheless, animals that were vaccinated with the rBCG-pBlaF promoter construct were able to develop a specific immunological response (Michelon et al. 2006). The pBlaF promoter drove the expression of Sm14 antigen from *Schistosoma mansoni* in rBCG, which was considerably stable and elicited a Th1-predominant immune response in vaccinated mice (Varaldo et al. 2004). Other studies that have used this promoter have also demonstrated its efficacy against diphtheria, *Bordetella pertussis*, and *Helicobacter pylori* infection (Lu et al. 2009; Miyaji et al. 2001; Nascimento et al. 2000).

Alpha antigen or fpbB promoter

The *alpha antigen*, also known as 30-kDa antigen or Ag85B, is a secreted protein that is widely distributed in slow-growing mycobacteria (Matsuo et al. 1988). The expression of MSP1 from *Plasmodium yoelii* under the control of the fbpB promoter has been found to result in protective immunity against infection (Matsumoto et al. 1998). In addition, overexpression of Ag85B using its own promoter has demonstrated superior efficacy to the conventional BCG (Horwitz et al. 2000; Rizzi et al. 2012). Joseph et al. found that the fbpB promoter was associated with low levels of HIV-1 gp120 gene expression. However, despite being considered a weak promoter, its use has been associated with a higher structural and functional stability (Joseph et al. 2010).

19 kDa The 19 kDa antigen is an immunodominant lipoprotein in *M. tuberculosis* (Garbe et al. 1993). In one study, the expression of OspA under the control of the 19 kDa promoter was found to result in lower levels of expression than using the Hsp60 promoter; however, the antibody response was 100–1000 times higher (Stover et al. 1993). These results could be attributed to the location of the antigen as a membrane-anchored lipoprotein. Similarly, the expression of MalE protein in BCG has been found to be lower when the 19 kDa promoter was used than it was when the pBlaF promoter was employed. However, there was no detectable antibody response in mice when the former construct was applied (Himmelrich et al. 2000).

Inducible promoters

A series of inducible expression systems are described for mycobacteria (Schnappinger and Ehrt 2014), and they are employed to study gene function and validate drug targets that employ conditional expression or knockdown strain generation (Blumenthal et al. 2010; Boldrin et al. 2014; Gandotra et al. 2007; Kaur et al. 2009; Kolly et al. 2014; Ravishankar et al. 2015) and overexpress mycobacterial proteins for biochemical, structural, or immunological studies (Chang et al. 2009; Leotta et al. 2015; Triccas et al. 1998). Alternately, BCG-expressing inducible genes can be used to enhance and maintain rBCG-induced immunity by modifying the antigen expression in the vaccine vehicle. The *hspX* promoter drives the *hspX* gene, which codifies the chaperone HspX or α-crystallin protein in *M. tuberculosis* and is upregulated in cultures that are grown under conditions of limited aeration and during the course of in vitro infection of macrophages (Yuan et al. 1998). Gene expression in BCG under the control of the *M. tuberculosis hspX* promoter has demonstrated rapid drive and the pronounced induction of gene expression by recombinant BCG within DCs, resulting in improved T cell priming and sustained generation of INF-gamma-secreting cells that recognize the recombinant antigen (Kong et al. 2011).

Engineered promoters

Engineering promoters represent a potent tool by which the expression of the recombinant antigen can be optimized, resulting in promoters of various strengths and altered regulatory profiles (Ruth and Glieder 2010). This approach employs techniques such as mutations on promoter sequences (Kanno et al. 2016; Oldfield and Hatfull 2014), regulatory sequences, or alteration of the initiation codon (Fan et al. 2009; Oldfield and Hatfull 2014). To obtain a set of mycobacterial promoters that offer predictable levels of expression of recombinant antigens in BCG, Kanno et al. recently subjected the P_{L5} promoter regulatory sequence to random mutagenesis by error-prone PCR (Kanno et al. 2016). They found that the P_{L5} promoters mutagenized allowed *S. mansoni* Sm29 antigen expression in BCG and the stable expression of enhanced green fluorescent protein (eGFP). The study also identified the promoter regions responsible for its strength.

In an alternative research study, mutations in the regulatory sequence and the promoter of the *furA* gene from *M. tuberculosis* generated a series of operator/promoter mutants, which were expressed continuously in BCG and induced early infection in macrophages (Fan et al. 2009). The *pfurA* promoter is an oxidative stress-inducible promoter that is always repressed by its own encoded FurA protein under normal growth conditions (Sala et al. 2003). The reporter *lacZ* gene was employed within the research, and the mutated sequence activities were analyzed by β-galactosidase assay. Mutations in FurA binding site, a conserved AT-rich region upstream of the *furA* gene, led to a four- to sixfold increase in β-galactosidase activity. Combined with a variation in the initial codon (GTG → ATG), the mutated promoter increased the β-galactosidase activity by approximately tenfold (Fan et al. 2009).

Oldfield and Hatfull generated mutated promoters by employing a site-directed mutagenesis technique on the P_R promoter from the mycobacteriophage BPs. Thus, a series of promoters was obtained that offered calibrated strengths and suitable intervals with regard to the expression of the mCherry reporter in *M. smegmatis* mc^2 and *M. tuberculosis* (Oldfield and Hatfull 2014). The P_R promoter was modulated by a repressor binding to its 12-pb operator (O_R), and the mutations revealed the positions that may play a prominent role in the repression activity (Broussard et al. 2013). Combining mutations in P_R that elevate transcription enables the construction of promoters that exhibit two times higher activity than the Hsp60 promoter in an integrated context (Oldfield and Hatfull 2014).

Location of foreign antigen

Recombinant BCG vaccines have been engineered to express the target antigen intracellularly, anchored-membrane and surface-exposed, or extracellularly (Table 1). The pathways for antigen presentation interfere in modulating the immune response that is subsequently induced (Grode et al. 2002). Fusion of the foreign antigen with mycobacterial signal sequences, such as from the *M. fortuitum* β-lactamase or the 19 kDa antigen, has allowed the protein to be delivered to the BCG surface (Garbe et al. 1993; Timm et al. 1994b). In addition, the secretion of foreign antigens has been engineered in some systems, which typically employ the signal sequence of the Ag85B protein (Matsuo et al. 1990).

Cytoplasm-targeted antigens

Proteins expressed cytoplasmically may present toxicity and, consequently, may have an impact on bacterial fitness. Moreover, the presence of cytoplasmic peptidases may be detrimental to the stable expression of heterologous antigens (Dennehy and Williamson 2005). Cytoplasm-targeted antigens will only be processed by MHC machinery after bacteria are killed by the macrophage. Thus, the intracellular location of the foreign antigen implies there is a delayed T-cell stimulation, especially for pathogens that survive and resist the intraphagosomal environment, as is the case with BCG (Kaufmann and Hess 1999). However, the delay in releasing and presenting the antigen may be useful for the induction of a long-lasting and sustained immunity, a feature that is also desirable for a vaccine.

Grode et al. demonstrated that BCG that expressed the p60 antigen from *Listeria monocytogenes* in cytosolic form failed to protect mice against listeriosis. However, when this antigen was secreted or membrane-anchored, an 80 and 100% protection respectively was observed (Grode et al. 2002). Similarly, the expression of PspA from *Streptococcus pneumoniae* in BCG elicited protective immunity when presented as a membrane-associated or secreted protein but not when directed to the cytoplasm (Langermann et al. 1994).

Cell membrane-targeted antigens

The mycobacteria cell wall consists of a peptidoglycan-glycolipid complex and a glycolipid fractions and is well known for its adjuvant properties (Bastos et al. 2009). The expression of bacterial wall-anchored proteins may represent a promising mechanism by which the immune response directed against these antigens can be improved (Grode et al. 2002). The 19 kDa protein from *M. tuberculosis* is a lipoprotein, the signal sequence of which is commonly used to drive the expression of heterologous antigens to the BCG surface (Garbe et al. 1993). This protein is highly immunogenic and is processed by both MHC class I and MHC class II pathways (Neyrolles et al. 2001), suggesting that these responses may be achieved through the use of its acylation signal for the expression of foreign antigens.

In one study, the expression of OspA antigen from *Borrelia burgdorferi* attached to cell membrane resulted in a protective immunity that was not observed for cytosolic or secreted expression (Stover et al. 1993). Similarly, using the same MT19 signal sequence, an rBCG strain was constructed to direct the expression of GP5 and M proteins from porcine reproductive respiratory syndrome virus (PRRSV) to the mycobacterial surface (Bastos et al. 2002). Mice inoculated with this rBCG strain developed antibodies that could neutralize PRRSV. However, when these antigens were expressed in the BCG cytoplasm, no antibody response was observed (Bastos et al. 2002).

The *blaF* gene encodes a class A beta-lactamase from *M. fortuitum*, exported beyond the cytoplasm to hydrolyze β-lactams antibiotics that act in the bacteria cell wall. The signal sequence of BlaF antigen is also used to drive the expression of foreign antigens in BCG (Lim et al. 1995; Timm et al. 1994b). In one study, the expression of pertussis toxin subunit S1 on the BCG cell wall, using the BlaF leader signal, resulted in the induction of Th1 immunity and protected mice against *B. pertussis* (Nascimento et al. 2000).

Secreted antigens

Secreted antigens are processed by the MHC before antigens are encapsulated in the bacteria cell (Kaufmann and Hess 1999). The signal sequence of the Ag85B protein is the sequence that is most commonly used to secrete heterologous proteins in BCG (Grode et al. 2005; Horwitz et al. 2000; Rizzi et al. 2012; Sun et al. 2009). The Ag85B is part of a complex of secretory proteins that also include the 85A and 85C antigens, which are the most abundant secreted proteins in mycobacteria (Wiker and Harboe 1992).

Table 1 Signal sequences most used for exportation of heterologous antigens in BCG

Name	Gene number	Gene name	Sequence	Size	Derived from	Form of antigen display	References
α antigen	Mb1918c	*fbpB*	MTDVSRKIRAWGRRLMIGTAAAVVLPGLVGLAGGAATAGA	40 aa	*M. bovis* BCG	Secreted	Grode et al. (2005)
α antigen	–	*fbpB*	MTDVSGKIRAWGRRLLVGAAAAAALPGLVGLAGGAATAGA	40 aa	*M. kanasii*	Secreted	Matsumoto et al. (1998)
α antigen	Rv1886c	*fbpB*	MTDVSRKIRAWGRRLMIGTAAAVVLPGLVGLAGGAATAGA	40 aa	*M. tuberculosis*	Secreted	Hart et al.(2015)
85A antigen	Rv3804c	*fbpA*	MQLVDRVRGAVTGMSRRLVVGAVGAALVSGLVGAVGGTATAGA	43 aa	*M. tuberculosis*	Secreted	Al-Zarouni and Dale (2002)
Erp antigen	Rv3810	*pirG*	MPNRRRRKLSTAMSAVAALAVA	22 aa	*M. tuberculosis*	Secreted	Himmelrich et al. (2000)
19 kDa lipoprotein	Rv3763	*lpqH*	MKRGLTVAVAGAAILVAGLSGCSSNKSTTGSGETTTAAGTTASPGA	46 aa	*M. tuberculosis*	Membrane-anchored	Bastos et al. (2002); Hart et al. (2015); Stover et al. (1993)
β-lactamase	–	*blaF*	MTGLSRRNVLIGSLVAAAAVGAGVGGAAPAFA	32 aa	*M. fortuitum*	Membrane-anchored	Lim et al. (1995); Nascimento et al. (2000); Timm et al. (1994b)

Al-Zarouni and Dale observed that the use of the 85A secretion signal to direct the expression of β-lactamase resulted in much higher levels of expression than the use of the 19-kDa signal to drive the expression to the membrane (Al-Zarouni and Dale 2002). This was attributed to the potential role the 85A signal sequence played in enhancing the transcription or stabilizing the protein during the export process. Matsumoto et al. constructed a BCG strain that secreted the MSP1 protein from *P. yoelii* through the use of the Ag85B signal sequence from *M. kanasii* (Matsumoto et al. 1998). Mice inoculated with this strain were protected against malaria infection.

Himmelrich et al. compared the expression of the MalE protein from *Escherichia coli* in BCG under different forms of antigen display (Himmelrich et al. 2000). The highest levels of MalE were obtained with the rBCG strain, the expression of which was secreted and controlled by the use of Erp antigen signal sequence. Similarly, the humoral and cellular immune response was also earlier and stronger in the secreting strain than in those expressing MalE protein in the cytoplasm or on the BCG membrane (Himmelrich et al. 2000). The Erp (exported repeated protein) antigen is an exported (Berthet et al. 1995) and conserved antigen among *Mycobacterium* species (de Mendonca-Lima et al. 2001). It induces a T-cell response and IgG3 production in individuals with latent tuberculosis (Martinez et al. 2007).

Moreover, the expression of cytolysins, such as listeriolysin O from *L. monocytogenes* and perfringolysin O from *Clostridium perfringens*, have been used as a strategy by which the access of antigens to the MHC I processing pathway can be improved and, consequently, CD8 T cells can be activated (Grode et al. 2005; Sun et al. 2009). Listeriolysin acts through the perforation of the phagosomal membrane, promoting the antigen translocation from the phagosome to the cytosol of macrophages (Nasser Eddine and Kaufmann 2005). Therefore, antigens are delivered into both processing pathways, MHC class I and II.

Stability of the vectors

Two types of genetic systems can be employed to obtain rBCG: integrative or episomal vectors. While integrative vectors have demonstrated improved stability and persistent synthesis of the target antigen in vivo, episomal vectors generally provide higher levels of expression (Bastos et al. 2009). However, the presence of antibiotic resistance genes as a selective agent in episomal vectors compromises their stability and has a negative effect on the obtainment of long-term immunity. The use of auxotrophic mutants has been described as an alternative strategy that can overcome these limitations (Borsuk et al. 2007; Chapman et al. 2012; Nascimento et al. 2009). Furthermore, the expression of recombinant antigens in BCG imposes a metabolic load and, in some cases, toxicity. This may reduce the bacterial fitness and result in the structural or functional loss of the heterologous antigen.

Integrative vectors

The insertional cloning of foreign genes is based on site-specific integration systems that are derived from mycobacteriophages, such as L517 or Ms6. Integrative vectors carry the integrase gene and *attP* site, which allow a single predictable recombination event with the bacteria chromosome (Hatfull 2014). These vectors are usually maintained in a single copy in the mycobacteria genome, which leads to lower expression levels, and, therefore, to a lower metabolic load (Bastos et al. 2009). It is likely that this reduced metabolic burden allows the persistent synthesis of the foreign antigen in vivo, providing more stability than that achieved when episomal vectors are used (Table 2). Moreover, integrative rBCG strains offer another advantage in comparison to replicative strains: there is no risk of vector horizontal transfer between other pathogens, thereby improving the safety of BCG-based live vaccines.

In one study, the integrative cloning of genes from the simian immunodeficiency virus in rBCG showed an 85–98% rate of stability in vivo after 100 days of inoculation, compared with only 25% when a replicative plasmid was used (Mederle et al. 2002). In addition, the IFN-gamma production induced after a single rBCG dose was only detected 6 weeks after inoculation in mice that had been vaccinated with the integrative rBCG strain. These results highlight the importance of a sustained and stable expression of the foreign antigen for achieving a long-lasting immunity.

New integrative vectors have been studied that aim to overcome the drawbacks of site-specific recombination using integrase derived from phage L5, particularly the unfavorable transcription of integrated genes (Murry et al. 2005). Murry et al. established a φC31 recombinase system that was derived from a bacteriophage that infected *Streptomyces coelicolor*, for mycobacteria. This system was efficient and stable in both *M. smegmatis* and *M. bovis* BCG; however, excision products were detected, and the level of recombination was relatively low (Murry et al. 2005). Excision was also reported in an L5-based integrating vector. Nevertheless, for L5 vectors, a strategy based on providing the *attP* site in *cis* and the integrase function in *trans* to prevent excision and instability was examined (Springer et al. 2001). To overcome the problems with excision, single-copy integration, and low levels of expression, Huff et al. constructed a system for multiple-site integration in mycobacteria genome, providing higher levels of expression than that on offer in single-copy integration systems (Huff et al. 2010).

Episomal vectors

The vast majority of extrachromosomal genetic systems developed for the expression of foreign antigens in BCG have used the origin of replication derived from pAL5000, a *M. fortuitum* plasmid (Bastos et al. 2009). The pAL5000-derived shuttle vectors allow approximately five copies of plasmid per cell, resulting in higher levels of expression than those achievable with integrative vectors (Bastos et al. 2009). A high-copy-number version of this replicon that maintained approximately 35 copies per cell was developed by Bourn et al. (Bourn et al. 2007). More recently, a novel *Mycobacterium-E. coli* shuttle vector system was developed using pMyong2 plasmid from *M. yongonense*. The pMyong2 vector allowed 37 times higher copies, and the expression levels of heterologous antigen increased 50-fold compared to pAL5000 (Lee et al. 2015).

Despite the high levels of recombinant proteins expressed using episomal vectors, the reported stability in vitro and in vivo using this system is poor (Table 2). Genetic instability may be detrimental to the induction of long-lasting immunity (Mederle et al. 2002). Plasmid stability is mainly affected by the selective pressure that is often associated with antibiotics resistance, which is lost in vivo. It is worth noting that, in one phase I clinical trial, a replicative rBCG strain expressing the OspA antigen from *B. burgdorferi*, the protective efficacy of which had already been demonstrated in mice, failed to induce a specific humoral response against OspA (Edelman et al. 1999). This failure was partially attributed to the plasmid loss in vivo, due to its high instability. The stability of plasmid is normally determined by the percentage of bacteria that retain antibiotic resistance, which is indicative of the presence of the recombinant plasmid (Dennehy and Williamson 2005). However, this is not mandatorily a reliable indicator of antigen expression. Therefore, systems that use auxotrophic complementation represent an alternative method by which in vivo selection can be maintained (Borsuk et al. 2007; Chapman et al. 2012; Nascimento et al. 2009). Borsuk et al. constructed a BCG auxotrophic for the amino acid leucine (BCG Δ*leuD*), which is able to replicate inside macrophages only in the presence of a replicative vector, pUP410, which supplements this mutation (Borsuk et al. 2007). Using this system, Seixas et al. determined the stability in vivo of vectors containing genes from *L. interrogans* (Seixas et al. 2010). After 18 weeks of inoculation, 100% of the BCG Δ*leuD* colonies harbored the recombinant plasmids while, in BCG Pasteur, only 7% of the colonies retained kanamycin resistance. However, the retention of antigen production was not evaluated in this study. Similarly, Nascimento et al. developed a system for the expression of a pertussis antigen based on a BCG lysine auxotrophic and a complementation vector. This system was able to maintain similar features to the original non-auxotrophic strain, including antigen expression level, immune response, and protection against challenge (Nascimento et al. 2009).

Using a leucine auxotrophic system for the expression of lentiviral antigens in BCG, Hart et al. demonstrated the vaccine stability and post-production quality control in terms of retention of plasmid, antigen expression, and immunogenicity

Table 2 Stability of recombinant BCG vaccine strains

Antigen (s)	Derived from	Genetic system	Percentage of stability (period after inoculation): Retention of selective marker – In vitro	Retention of selective marker – In vivo	Retention of antigen production – In vitro	Retention of antigen production – In vivo	References
LacZ	*Escherichia coli*	E	ND	100% (2–4 weeks)	ND	100% (2–4 weeks)	Stover et al. (1991)
β-galactosidase	*Escherichia coli*	E	ND	45–46% (2 months)	ND	26–27% (2 months)	Murray et al. (1992)
LacZ	*Escherichia coli*	E	ND	~ 50% (8 weeks)	ND	~ 50% (16 weeks)	Lagranderie et al. (1993)
OspA	*Borrelia burgdorferi*	E	ND	~ 18% (12 weeks)	ND	ND	Edelman et al. (1999)
S1 subunit of pertusis toxin	*Bordetella pertussis*	E	ND	85% (2 months)	ND	ND	Nascimento et al. (2000)
MSP1a	*Anaplasma marginale*	E	90% (7 days)	ND	100% (7 days)	ND	Michelon et al. (2006)
Ag85B-ESAT-6	*Mycobacterium tuberculosis*	E	ND	54% (58 days)	ND	ND	Badell et al. (2009)
gp63	*Leishmania major*	E	ND	100% (10 weeks)	ND	ND	Connell et al. (1993)
CSP	*Plasmodium falciparum*	I	100% (50 generations)	ND	Yes (44 days)	ND	Haeseleer et al. (1993)
Nucleocapsid protein	*Measles virus*	E	ND	ND	ND	100% (8 weeks)	Fennelly et al. (1995)
Nucleocapsid protein	*Measles virus*	E	ND	ND	ND	> 90% (2 weeks)	Zhu et al. 1997)
LCR1	*Leishmania chagasi*	E	ND	3% (8 weeks)	ND	ND	Streit et al. (2000)
Sm14	*Schistosoma mansoni*	E	90% (7 days)	ND	ND	ND	Varaldo et al. (2004)
Nef, Gag	*Simian immunodeficiency virus*	E	45% (60 generations)	25% (100 days)	ND	25% (70 days)	Mederle et al. (2002)
		I	~100% (100 generations)	85–98% (100 days)	ND	100% (70 days)	
Nef, Gag, Env	*Simian immunodeficiency virus*	E	ND	42–70% (4 weeks)	ND	ND	Mederle et al. (2003)
S antigens	*Hepatitis B virus*	E	100% (4 days)	48% (15 days)	ND	ND	Rezende et al. (2005)
VP6	*Rotavirus*	E	0–2% (30 generations)	ND	ND	ND	Dennehy et al. (2007)
		I	100% (30 generations)	ND	ND	ND	
lacZ	*Escherichia coli*	E*	100% (7 generations)	90–100% (30 weeks)	100% (6 generations)	100%	Borsuk et al. (2007)
LipL32, LigAni	*Leptospira interrogans*	E*	ND	100% (18 weeks)	ND	ND	Seixas et al. (2010)
Fusion protein CMX	*Mycobacterium tuberculosis*	E	ND	~100% (15 days)	ND	ND	da Costa et al. (2014a)
Gp 120	*HIV*	E	ND	ND	100% (12 weeks)	100% (60 days)	Hart et al. (2015)
SIV gag	*Simian immunodeficiency virus*	E	ND	ND	100% (29 weeks)	100% (30 days)	Hart et al. (2015)
smGFP	*Aequorea victoria*	E	50–66% (42 generations)	ND	86–92% (42 generations)	ND	Griffin et al. (2009)

Appl Microbiol Biotechnol (2017) 101:6865–6877

Table 2 (continued)

Antigen (s)	Derived from	Genetic system	Percentage of stability (period after inoculation): Retention of selective marker		Retention of antigen production		References
			In vitro	In vivo	In vitro	In vivo	
HspX	*Mycobacterium tuberculosis*	E	ND	ND	100% (4 months)	ND	Shi et al. (2010)
Rv1767	*Mycobacterium tuberculosis*	E	ND	65–99% (8 weeks)	ND	100% (4 weeks)	Speranza et al. (2010)
Gag p24	*HIV-1*	E	66–96% (40 generations)	ND	100% (70 generations)	ND	Chapman et al. (2014)
clade A immunogen HIVA	*HIV*	E[a]	100% (30 generations)	100% (7 weeks)	80% (30 generations)	ND	Saubi et al. (2014)
envelop glycoprotein V3	*HIV-1*	E	ND	100% (8 weeks)	100% (4 weeks)	100% (8 weeks)	Kim (2011)

E episomal, *I* integrative, *ND* not determined

[a] Auxotrophic strain

(Hart et al. 2015). The expression of lentiviral antigens was maintained in vivo for at least 60 days; as such, this system may contribute to the induction of a long-lasting immune response. Thus, these systems maintain the high levels of expression of the foreign antigen and the selective pressure in vivo, whereas they avoid the use of antibiotic resistance genes as selectable markers, thereby improving vaccine safety.

The use of reporter systems to investigate molecular factors in rBCG

Expression analysis in rBCG is especially important for determining the functionality of new genetic systems in vitro and in vivo. Moreover, monitoring expression levels is a valuable tool to assay promoter activity, which is strongly associated with the stability of rBCG strains. It is well known that the promoter activity and, consequently, the levels of expression can vary in vitro and in vivo (Dellagostin et al. 1995). Therefore, the internalization of mycobacteria inside macrophages is essential to access the intracellular expression of heterologous genes, simulating in vivo conditions. These analyses are usually performed using reporter systems, such as GFP and β-galactosidase. Dellagostin et al. elucidated the activation pattern of different promoters inside macrophages through assay of β-galactosidase activity (Dellagostin et al. 1995). Once the β-galactosidase enzyme is stable and absent in mycobacteria, it represents a valuable reporter system that avoids background reactions (Carroll and James 2009). Furthermore, through using β-galactosidase, Fan et al. evaluated, in vitro and during macrophage infection, the activity of a range of mutated promoters that were derived from the *M. tuberculosis furA* gene promoter region. Thus, they generated a series of vectors for the expression of target antigens at different levels and demonstrated that the system could feasibly modulate and control gene expression in rBCG (Fan et al. 2009).

Fluorescent proteins have been extensively used as reporter systems in rBCG. Eitson et al. developed shuttle vectors to generate a high level of protein expression. Using GFP as a reporter gene, they assessed expression levels in vitro and in infected macrophages through flow cytometry (Eitson et al. 2012). Similarly, fluorescent proteins were also used to characterize a high-copy vector system derived from *M. yongonense* in terms of its stability, compatibility with the pAL5000 vector system, and ability to express foreign antigens in vitro and in vivo (Lee et al. 2015). As mentioned above, Kanno et al. selected a group of mutagenized promoters that were derived from the pL5 promoter, and then used the eGFP to evaluate the strength of these promoters in *M. smegmatis* and *M. bovis* BCG. Thus, they established a rational and predictable system by which antigens could be expressed in mycobacteria (Kanno et al. 2016). However, the activity of these promoters must also be measured within macrophages. An integrative fluorescent reporter system was developed to study promoter activity in vitro and in vivo (Roy et al. 2012), which is usually evaluated using episomal vectors, as reviewed above. A reporter system that employed Cherry fluorescent protein was also constructed to study mycobacteria transcriptional machinery in *E. coli* (Banerjee et al. 2015).

Interestingly, reporter strains of *M. tuberculosis* were used to study how mycobacteria adapted in vaccinated and non-vaccinated host immune environments. Using a reporter strain that expressed GFP under the control of the inducible promoter *hspX*, the research findings demonstrated that there were higher levels of fluorescence in the *M. tuberculosis* present in vaccinated mice than there was in unvaccinated mice at 14 days post-infection (Sukumar et al. 2014). These results are consistent with an adaptive immunity pre-existing in vaccinated animals, which is responsible for early macrophage activation to produce anti-microbial molecules, such as NO, an inducer for the *hspX* promoter. Bioluminescent strains, usually expressing luciferases, have also been successfully used to assess the efficacy of antimycobacterial compounds and vaccines (Andreu et al. 2013; Kampmann et al. 2004; Shawar et al. 1997; Zhang et al. 2012). Singh et al. established a platform for a selective screening of antimycobacterial compounds, through the expression of two reporter genes in rBCG, under the control of two different promoters: one constitutive and the other inducible by FAS-II pathway inhibitors (Singh et al. 2014). Recently, new fluorescent proteins have been used as reporters to measure the anti-TB therapeutic efficacy of rifampicin and isoniazid in vitro and in vivo (Kong et al. 2016).

These findings highlight the important role reporter systems play in characterizing new genetic systems and demonstrating their functionality in mycobacteria. Moreover, these systems represent a reliable tool by which several factors can be investigated, such as gene modulation, transcriptional regulation, dynamic of infection, drug screening assays, evaluation of therapeutics, and their impact on vaccination and immunity.

Conclusion

When used in immunoprophylaxis and immunotherapy, *M. bovis* BCG remains a promising vehicle for the expression of homologous or heterologous antigens. Understanding the regulatory mechanisms at the transcriptional and post-transcriptional levels is primordial to predicting the efficacy of the rBCG in terms of fitness, production rate, toxicity of the target protein, and stability. This review summarized a range of molecular factors, including promoters, signal sequences, and genetic systems, which are crucial for the genetic control of rBCG and the subsequent immune response against the target antigen. Further research related to engineered and inducible promoters, codon optimization, and novel vector systems will help to identify the methods by which the variables that directly affect the immunogenicity of rBCG can be optimized.

Acknowledgements This work was funded by grants from CNPq, CAPES, and FAPERGS, Brazilian Research Funding Agencies.

Compliance with ethical standards

Funding This study is funded by CNPq and CAPES (grant number 482376/2010-4). The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abdelhak S, Louzir H, Timm J, Blel L, Benlasfar Z, Lagranderie M, Gheorghiu M, Dellagi K, Gicquel B (1995) Recombinant BCG expressing the leishmania surface antigen Gp63 induces protective immunity against *Leishmania major* infection in BALB/c mice. Microbiology 141(Pt 7):1585–1592. doi:10.1099/13500872-141-7-1585

Al-Zarouni M, Dale JW (2002) Expression of foreign genes in Mycobacterium bovis BCG strains using different promoters reveals instability of the hsp60 promoter for expression of foreign genes in *Mycobacterium bovis* BCG strains. Tuberculosis (Edinb) 82(6): 283–291

Andreu N, Zelmer A, Sampson SL, Ikeh M, Bancroft GJ, Schaible UE, Wiles S, Robertson BD (2013) Rapid in vivo assessment of drug efficacy against *Mycobacterium tuberculosis* using an improved firefly luciferase. J Antimicrob Chemother 68(9):2118–2127. doi: 10.1093/jac/dkt155

Badell E, Nicolle F, Clark S, Majlessi L, Boudou F, Martino A, Castello-Branco L, Leclerc C, Lewis DJ, Marsh PD, Gicquel B, Winter N (2009) Protection against tuberculosis induced by oral prime with *Mycobacterium bovis* BCG and intranasal subunit boost based on the vaccine candidate Ag85B-ESAT-6 does not correlate with circulating IFN-gamma producing T-cells. Vaccine 27(1):28–37. doi:10.1016/j.vaccine.2008.10.034

Banerjee R, Rudra P, Saha A, Mukhopadhyay J (2015) Recombinant reporter assay using transcriptional machinery of *Mycobacterium tuberculosis*. J Bacteriol 197(3):646–653. doi:10.1128/JB.02445-14

Bastos RG, Borsuk S, Seixas FK, Dellagostin OA (2009) Recombinant *Mycobacterium bovis* BCG. Vaccine 27(47):6495–6503. doi:10.1016/j.vaccine.2009.08.044

Bastos RG, Dellagostin OA, Barletta RG, Doster AR, Nelson E, Osorio FA (2002) Construction and immunogenicity of recombinant *Mycobacterium bovis* BCG expressing GP5 and M protein of porcine reproductive respiratory syndrome virus. Vaccine 21(1–2):21–29

Batoni G, Maisetta G, Florio W, Freer G, Campa M, Senesi S (1998) Analysis of the *Mycobacterium bovis* hsp60 promoter activity in recombinant *Mycobacterium avium*. FEMS Microbiol Lett 169(1): 117–124

Batra B (2012) Heat shock proteins in *Mycobacterium tuberculosis*: involvement in survival and virulence of the pathogen. In: Cardona DP-J (ed) Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli InTech, pp 257–276

Berthet FX, Rauzier J, Lim EM, Philipp W, Gicquel B, Portnoi D (1995) Characterization of the *Mycobacterium tuberculosis* erp gene encoding a potential cell surface protein with repetitive structures. Microbiology 141(Pt 9):2123–2130. doi:10.1099/13500872-141-9-2123

Blumenthal A, Trujillo C, Ehrt S, Schnappinger D (2010) Simultaneous analysis of multiple *Mycobacterium tuberculosis* knockdown mutants in vitro and in vivo. PLoS One 5(12):e15667. doi:10.1371/journal.pone.0015667

Boldrin F, Ventura M, Degiacomi G, Ravishankar S, Sala C, Svetlikova Z, Ambady A, Dhar N, Kordulakova J, Zhang M, Serafini A, Vishwas KG, Kolly GS, Kumar N, Palu G, Guerin ME, Mikusova K, Cole ST, Manganelli R (2014) The phosphatidyl-myo-inositol mannosyltransferase PimA is essential for *Mycobacterium tuberculosis* growth in vitro and in vivo. J Bacteriol 196(19):3441–3451. doi:10.1128/JB.01346-13

Borsuk S, Mendum TA, Fagundes MQ, Michelon M, Cunha CW, McFadden J, Dellagostin OA (2007) Auxotrophic complementation as a selectable marker for stable expression of foreign antigens in *Mycobacterium bovis* BCG. Tuberculosis (Edinb) 87(6):474–480. doi:10.1016/j.tube.2007.07.006

Bourn WR, Jansen Y, Stutz H, Warren RM, Williamson AL, van Helden PD (2007) Creation and characterisation of a high-copy-number version of the pAL5000 mycobacterial replicon. Tuberculosis (Edinb) 87(6):481–488. doi:10.1016/j.tube.2007.08.003

Bretl DJ, Demetriadou C, Zahrt TC (2011) Adaptation to environmental stimuli within the host: two-component signal transduction systems of *Mycobacterium tuberculosis*. Microbiol Mol Biol Rev 75(4):566–582. doi:10.1128/MMBR.05004-11

Broussard GW, Oldfield LM, Villanueva VM, Lunt BL, Shine EE, Hatfull GF (2013) Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Mol Cell 49(2):237–248. doi:10.1016/j.molcel.2012.11.012 Epub 2012 Dec 13

Carroll P, James J (2009) Assaying promoter activity using LacZ and GFP as reporters. Methods Mol Biol 465:265–277. doi:10.1007/978-1-59745-207-6_18

Chang Y, Mead D, Dhodda V, Brumm P, Fox BG (2009) One-plasmid tunable coexpression for mycobacterial protein-protein interaction studies. Protein Sci 18(11):2316–2325. doi:10.1002/pro.242

Chapman R, Bourn WR, Shephard E, Stutz H, Douglass N, Mgwebi T, Meyers A, Chin'ombe N, Williamson AL (2014) The use of directed evolution to create a stable and immunogenic recombinant BCG expressing a modified HIV-1 gag antigen. PLoS One 9(7):e103314. doi:10.1371/journal.pone.0103314

Chapman R, Shephard E, Stutz H, Douglass N, Sambandamurthy V, Garcia I, Ryffel B, Jacobs W, Williamson AL (2012) Priming with a recombinant pantothenate auxotroph of *Mycobacterium bovis* BCG and boosting with MVA elicits HIV-1 gag specific CD8+ T cells. PLoS One 7(3):e32769. doi:10.1371/journal.pone.0032769

Cheng J, Huangfu Y, Feng Z, Liang J, Xiao H (1997) Expression of foreign gene in mycobacterium regulated by human *Mycobacterium tuberculosis* heat shock protein 70 promoter. J Tongji Med Univ 17(4):193–199

Cockle PJ, Gordon SV, Lalvani A, Buddle BM, Hewinson RG, Vordermeier HM (2002) Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. Infect Immun 70(12):6996–7003

Connell ND, Medina-Acosta E, McMaster WR, Bloom BR, Russell DG (1993) Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. Proc Natl Acad Sci U S A 90(24):11473–11477

da Costa AC, Costa-Junior Ade O, de Oliveira FM, Nogueira SV, Rosa JD, Resende DP, Kipnis A, Junqueira-Kipnis AP (2014a) A new recombinant BCG vaccine induces specific Th17 and Th1 effector cells with higher protective efficacy against tuberculosis. PLoS One 9(11):e112848. doi:10.1371/journal.pone.0112848

da Costa AC, Nogueira SV, Kipnis A, Junqueira-Kipnis AP (2014b) Recombinant BCG: innovations on an old vaccine. Scope of BCG strains and strategies to improve long-lasting memory. Front Immunol 5:–152. doi:10.3389/fimmu.2014.00152

de Mendonca-Lima L, Picardeau M, Raynaud C, Rauzier J, de la Salmoniere YO, Barker L, Bigi F, Cataldi A, Gicquel B, Reyrat JM (2001) Erp, an extracellular protein family specific to mycobacteria. Microbiology 147(Pt 8):2315–2320. doi:10.1099/00221287-147-8-2315

Dellagostin OA, Esposito G, Eales LJ, Dale JW, McFadden J (1995) Activity of mycobacterial promoters during intracellular and extracellular growth. Microbiology 141(Pt 8):1785–1792. doi:10.1099/13500872-141-8-1785

Dennehy M, Bourn W, Steele D, Williamson AL (2007) Evaluation of recombinant BCG expressing rotavirus VP6 as an anti-rotavirus vaccine. Vaccine 25(18):3646–3657. doi:10.1016/j.vaccine.2007.01.087

Dennehy M, Williamson AL (2005) Factors influencing the immune response to foreign antigen expressed in recombinant BCG vaccines. Vaccine 23(10):1209–1224. doi:10.1016/j.vaccine.2004.08.039

Edelman R, Palmer K, Russ KG, Secrest HP, Becker JA, Bodison SA, Perry JG, Sills AR, Barbour AG, Luke CJ, Hanson MS, Stover CK, Burlein JE, Bansal GP, Connor EM, Koenig S (1999) Safety and immunogenicity of recombinant Bacille Calmette-Guerin (rBCG) expressing *Borrelia burgdorferi* outer surface protein A (OspA) lipoprotein in adult volunteers: a candidate Lyme disease vaccine. Vaccine 17(7–8):904–914

Eitson JL, Medeiros JJ, Hoover AR, Srivastava S, Roybal KT, Ainsa JA, Hansen EJ, Gumbo T, van Oers NS (2012) Mycobacterial shuttle vectors designed for high-level protein expression in infected macrophages. Appl Environ Microbiol 78(19):6829–6837. doi:10.1128/AEM.01674-12

Fan XY, Ma H, Guo J, Li ZM, Cheng ZH, Guo SQ, Zhao GP (2009) A novel differential expression system for gene modulation in *Mycobacteria*. Plasmid 61(1):39–46. doi:10.1016/j.plasmid.2008.09.002

Fennelly GJ, Flynn JL, ter Meulen V, Liebert UG, Bloom BR (1995) Recombinant bacille Calmette-Guerin priming against measles. J Infect Dis 172(3):698–705

Gandotra S, Schnappinger D, Monteleone M, Hillen W, Ehrt S (2007) In vivo gene silencing identifies the *Mycobacterium tuberculosis* proteasome as essential for the bacteria to persist in mice. Nat Med 13(12):1515–1520 Epub 2007 Dec 2

Garbe T, Harris D, Vordermeier M, Lathigra R, Ivanyi J, Young D (1993) Expression of the *Mycobacterium tuberculosis* 19-kilodalton antigen in *Mycobacterium smegmatis*: immunological analysis and evidence of glycosylation. Infect Immun 61(1):260–267

Gormley E, Sandall L, Hong C, Lawton D, Murray A (1997) Identification and differentiation of mycobacteria using the PAN promoter sequence from *Mycobacterium paratuberculosis* as a DNA probe. FEMS Microbiol Lett 147(1):63–68

Govan VA, Christensen ND, Berkower C, Jacobs WR Jr, Williamson AL (2006) Immunisation with recombinant BCG expressing the cottontail rabbit papillomavirus (CRPV) L1 gene provides protection from CRPV challenge. Vaccine 24(12):2087–2093. doi:10.1016/j.vaccine.2005.11.029

Griffin S, Williamson AL, Chapman R (2009) Optimisation of a mycobacterial replicon increases foreign antigen expression in mycobacteria. Tuberculosis (Edinb) 89(3):225–232. doi:10.1016/j.tube.2009.03.006

Grode L, Kursar M, Fensterle J, Kaufmann SH, Hess J (2002) Cell-mediated immunity induced by recombinant *Mycobacterium bovis* Bacille Calmette-Guerin strains against an intracellular bacterial pathogen: importance of antigen secretion or membrane-targeted antigen display as lipoprotein for vaccine efficacy. J Immunol 168(4):1869–1876

Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, Mann P, Goosmann C, Bandermann S, Smith D, Bancroft GJ, Reyrat JM, van Soolingen D, Raupach B, Kaufmann SH (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest 115(9):2472–2479. doi:10.1172/JCI24617

Haeseleer F, Pollet JF, Haumont M, Bollen A, Jacobs P (1993) Stable integration and expression of the *Plasmodium falciparum* circumsporozoite protein coding sequence in mycobacteria. Mol Biochem Parasitol 57(1):117–126

Hart BE, Asrican R, Lim SY, Sixsmith JD, Lukose R, Souther SJ, Rayasam SD, Saelens JW, Chen CJ, Seay SA, Berney-Meyer L, Magtanong L, Vermeul K, Pajanirassa P, Jimenez AE, Ng TW, Tobin DM, Porcelli SA, Larsen MH, Schmitz JE, Haynes BF, Jacobs WR Jr, Lee S, Frothingham R (2015) Stable expression of lentiviral antigens by quality-controlled recombinant *Mycobacterium bovis* BCG vectors. Clin Vaccine Immunol 22(7): 726–741. doi:10.1128/CVI.00075-15

Hatfull GF (2014) Molecular genetics of Mycobacteriophages. Microbiol Spectr 2(2):1–36

Himmelrich H, Lo-Man R, Winter N, Guermonprez P, Sedlik C, Rojas M, Monnaie D, Gheorghiu M, Lagranderie M, Hofnung M, Gicquel B, Clement JM, Leclerc C (2000) Immune responses induced by recombinant BCG strains according to level of production of a foreign antigen: malE. Vaccine 18(24):2636–2647

Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S (2000) Recombinant bacillus calmette-guerin (BCG) vaccines expressing the mycobacterium tuberculosis 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. Proc Natl Acad Sci U S A 97(25):13853–13858. doi:10.1073/pnas.250480397250480397

Huff J, Czyz A, Landick R, Niederweis M (2010) Taking phage integration to the next level as a genetic tool for mycobacteria. Gene 468(1–2):8–19. doi:10.1016/j.gene.2010.07.012

Joseph J, Fernandez-Lloris R, Pezzat E, Saubi N, Cardona PJ, Mothe B, Gatell JM (2010) Molecular characterization of heterologous HIV-1gp120 gene expression disruption in *Mycobacterium bovis* BCG host strain: a critical issue for engineering mycobacterial based-vaccine vectors. J Biomed Biotechnol 2010:357370. doi:10.1155/2010/357370

Kampmann B, Tena GN, Mzazi S, Eley B, Young DB, Levin M (2004) Novel human in vitro system for evaluating antimycobacterial vaccines. Infect Immun 72(11):6401–6407. doi:10.1128/IAI.72.11.6401-6407.2004

Kanno AI, Goulart C, Rofatto HK, Oliveira SC, Leite LC, McFadden J (2016) New recombinant *Mycobacterium bovis* BCG expression vectors: improving genetic control over mycobacterial promoters. Appl Environ Microbiol 82(8):2240–2246. doi:10.1128/AEM.03677-15

Kaufmann SH, Hess J (1999) Impact of intracellular location of and antigen display by intracellular bacteria: implications for vaccine development. Immunol Lett 65(1–2):81–84

Kaur P, Agarwal S, Datta S (2009) Delineating bacteriostatic and bactericidal targets in mycobacteria using IPTG inducible antisense expression. PLoS One 4(6):e5923. doi:10.1371/journal.pone.0005923

Kim YJ (2011) Efficiency of recombinant bacille Calmette-Guerin in inducing humoral and cell mediated immunities against human immunodeficiency virus type 1 third variable domain in immunized mice. Yonsei Med J 52(1):173–180. doi:10.3349/ymj.2011.52.1.173

Kolly GS, Boldrin F, Sala C, Dhar N, Hartkoorn RC, Ventura M, Serafini A, McKinney JD, Manganelli R, Cole ST (2014) Assessing the es- sentiality of the decaprenyl-phospho-d-arabinofuranose pathway in *Mycobacterium tuberculosis* using conditional mutants. Mol Microbiol 92(1):194–211. doi:10.1111/mmi.12546 Epub 2014 Mar 7

Kong CU, Ng LG, Nambiar JK, Spratt JM, Weninger W, Triccas JA (2011) Targeted induction of antigen expression within dendritic cells modulates antigen-specific immunity afforded by recombinant BCG. Vaccine 29(7):1374–1381. doi:10.1016/j.vaccine.2010.12.070

Kong D, Kunimoto DY (1995) Secretion of human interleukin 2 by recombinant *Mycobacterium bovis* BCG. Infect Immun 63(3): 799–803

Kong Y, Yang D, Cirillo SL, Li S, Akin A, Francis KP, Maloney T, Cirillo JD (2016) Application of fluorescent protein expressing strains to evaluation of anti-tuberculosis therapeutic efficacy in vitro and in vivo. PLoS One 11(3):e0149972. doi:10.1371/journal.pone.0149972

Lagranderie M, Lo-Man R, Deriaud E, Gicquel B, Gheorghiu M, Leclerc C (1997) Genetic control of antibody responses induced by recombinant *Mycobacterium bovis* BCG expressing a foreign antigen. Infect Immun 65(8):3057–3064

Lagranderie M, Murray A, Gicquel B, Leclerc C, Gheorghiu M (1993) Oral immunization with recombinant BCG induces cellular and humoral immune responses against the foreign antigen. Vaccine 11(13):1283–1290

Langermann S, Palaszynski SR, Burlein JE, Koenig S, Hanson MS, Briles DE, Stover CK (1994) Protective humoral response against pneumococcal infection in mice elicited by recombinant bacille Calmette-Guerin vaccines expressing pneumococcal surface protein A. J Exp Med 180(6):2277–2286

Lee H, Kim BJ, Kim BR, Kook YH (2015) The development of a novel *Mycobacterium-Escherichia coli* shuttle vector system using pMyong2, a linear plasmid from *Mycobacterium yongonense* DSM 45126T. PLoS One 10(3):e0122897. doi:10.1371/journal.pone.0122897

Leotta L, Spratt JM, Kong CU, Triccas JA (2015) An inducible expression system for high-level expression of recombinant proteins in slow growing mycobacteria. Plasmid 81:27–31. doi:10.1016/j.plasmid.2015.05.002

Liang J, Teng X, Yuan X, Zhang Y, Shi C, Yue T, Zhou L, Li J, Fan X (2015) Enhanced and durable protective immune responses induced by a cocktail of recombinant BCG strains expressing antigens of multistage of *Mycobacterium tuberculosis*. Mol Immunol 66(2): 392–401. doi:10.1016/j.molimm.2015.04.017

Lim EM, Rauzier J, Timm J, Torrea G, Murray A, Gicquel B, Portnoi D (1995) Identification of *Mycobacterium tuberculosis* DNA sequences encoding exported proteins by using phoA gene fusions. J Bacteriol 177(1):59–65

Lu L, Cao HD, Zeng HQ, Wang PL, Wang LJ, Liu SN, Xiang TX (2009) Recombinant *Mycobacterium smegmatis* mc(2)155 vaccine expressing outer membrane protein 26 kDa antigen affords therapeutic protection against *Helicobacter pylori* infection. Vaccine 27(7):972–978. doi:10.1016/j.vaccine.2008.12.003

Maheshwari JJ, Dharmalingam K (2013) Protective role of *Mycobacterium leprae* small heat-shock protein in heterologous hosts, *Escherichia coli* and *Mycobacterium smegmatis*, grown under stress. J Med Microbiol 62(Pt 7):959–967. doi:10.1099/jmm.0.057851-0

Martinez V, Carcelain G, Badell E, Jouan M, Mauger I, Sellier P, Truffot C, Bricaire F, Arend SM, Ottenhoff T, Autran B, Gicquel B (2007) T-cell and serological responses to Erp, an exported *Mycobacterium tuberculosis* protein, in tuberculosis patients and healthy individuals. BMC Infect Dis 7:83. doi:10.1186/1471-2334-7-83

Matsumoto S, Yukitake H, Kanbara H, Yamada T (1998) Recombinant *Mycobacterium bovis* bacillus Calmette-Guerin secreting merozoite surface protein 1 (MSP1) induces protection against rodent malaria parasite infection depending on MSP1-stimulated interferon gamma and parasite-specific antibodies. J Exp Med 188(5):845–854

Matsuo K, Yamaguchi R, Yamazaki A, Tasaka H, Terasaka K, Totsuka M, Kobayashi K, Yukitake H, Yamada T (1990) Establishment of a foreign antigen secretion system in mycobacteria. Infect Immun 58(12):4049–4054

Matsuo K, Yamaguchi R, Yamazaki A, Tasaka H, Yamada T (1988) Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular alpha antigen. J Bacteriol 170(9):3847–3854

Medeiros MA, Dellagostin OA, Armoa GR, Degrave WM, De Mendonca-Lima L, Lopes MQ, Costa JF, McFadden J, McIntosh D (2002) Comparative evaluation of *Mycobacterium vaccae* as a surrogate cloning host for use in the study of mycobacterial genetics. Microbiology 148(Pt 7):1999–2009. doi:10.1099/00221287-148-7-1999

Mederle I, Bourguin I, Ensergueix D, Badell E, Moniz-Peireira J, Gicquel B, Winter N (2002) Plasmidic versus insertional cloning of heterologous genes in *Mycobacterium bovis* BCG: impact on in vivo antigen persistence and immune responses. Infect Immun 70(1):303–314

Mederle I, Le Grand R, Vaslin B, Badell E, Vingert B, Dormont D, Gicquel B, Winter N (2003) Mucosal administration of three recombinant *Mycobacterium bovis* BCG-SIVmac251 strains to cynomolgus macaques induces rectal IgAs and boosts systemic cellular immune responses that are primed by intradermal vaccination. Vaccine 21(27–30):4153–4166

Michelon A, Conceicao FR, Binsfeld PC, da Cunha CW, Moreira AN, Argondizzo AP, McIntosh D, Armoa GR, Campos AS, Farber M, McFadden J, Dellagostin OA (2006) Immunogenicity of *Mycobacterium bovis* BCG expressing *Anaplasma marginale* MSP1a antigen. Vaccine 24(37–39):6332–6339. doi:10.1016/j.vaccine.2006.05.028

Miyaji EN, Mazzantini RP, Dias WO, Nascimento AL, Marcovistz R, Matos DS, Raw I, Winter N, Gicquel B, Rappuoli R, Leite LC (2001) Induction of neutralizing antibodies against diphtheria toxin by priming with recombinant *Mycobacterium bovis* BCG expressing CRM(197), a mutant diphtheria toxin. Infect Immun 69(2):869–874. doi:10.1128/IAI.69.2.869-874.2001

Murray A, Winter N, Lagranderie M, Hill DF, Rauzier J, Timm J, Leclerc C, Moriarty KM, Gheorghiu M, Gicquel B (1992) Expression of *Escherichia coli* beta-galactosidase in *Mycobacterium bovis* BCG using an expression system isolated from *Mycobacterium paratuberculosis* which induced humoral and cellular immune responses. Mol Microbiol 6(22):3331–3342

Murray PJ, Aldovini A, Young RA (1996) Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines. Proc Natl Acad Sci U S A 93(2):934–939

Murry J, Sassetti CM, Moreira J, Lane J, Rubin EJ (2005) A new site-specific integration system for mycobacteria. Tuberculosis (Edinb) 85(5–6):317–323. doi:10.1016/j.tube.2005.08.016

Nascimento IP, Dias WO, Mazzantini RP, Miyaji EN, Gamberini M, Quintilio W, Gebara VC, Cardoso DF, Ho PL, Raw I, Winter N, Gicquel B, Rappuoli R, Leite LC (2000) Recombinant *Mycobacterium bovis* BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live *Bordetella pertussis* in mice. Infect Immun 68(9):4877–4883

Nascimento IP, Dias WO, Quintilio W, Hsu T, Jacobs WR Jr, Leite LC (2009) Construction of an unmarked recombinant BCG expressing a pertussis antigen by auxotrophic complementation: protection against *Bordetella pertussis* challenge in neonates. Vaccine 27(52):7346–7351. doi:10.1016/j.vaccine.2009.09.043

Nasser Eddine A, Kaufmann SH (2005) Improved protection by recombinant BCG. Microbes Infect 7(5–6):939–946. doi:10.1016/j.micinf.2005.03.012

Newton-Foot M, Gey van Pittius NC (2013) The complex architecture of mycobacterial promoters. Tuberculosis (Edinb) 93(1):60–74. doi:10.1016/j.tube.2012.08.003

Neyrolles O, Gould K, Gares MP, Brett S, Janssen R, O'Gaora P, Herrmann JL, Prevost MC, Perret E, Thole JE, Young D (2001) Lipoprotein access to MHC class I presentation during infection of murine macrophages with live mycobacteria. J Immunol 166(1):447–457

Oldfield LM, Hatfull GF (2014) Mutational analysis of the mycobacteriophage BPs promoter PR reveals context-dependent sequences for mycobacterial gene expression. J Bacteriol 196(20):3589–3597. doi:10.1128/JB.01801-14

Ravishankar S, Ambady A, Ramu H, Mudugal NV, Tunduguru R, Anbarasu A, Sharma UK, Sambandamurthy VK, Ramaiah S (2015) An IPTG inducible conditional expression system for mycobacteria. PLoS One 10(8):e0134562. doi:10.1371/journal.pone.0134562

Rezende CA, De Moraes MT, De Souza Matos DC, McIntoch D, Armoa GR (2005) Humoral response and genetic stability of recombinant BCG expressing hepatitis B surface antigens. J Virol Methods 125(1):1–9. doi:10.1016/j.jviromet.2004.11.026

Rizzi C, Bianco MV, Blanco FC, Soria M, Gravisaco MJ, Montenegro V, Vagnoni L, Buddle B, Garbaccio S, Delgado F, Leal KS, Cataldi AA, Dellagostin OA, Bigi F (2012) Vaccination with a BCG strain overexpressing Ag85B protects cattle against *Mycobacterium bovis* challenge. PLoS One 7(12):e51396. doi:10.1371/journal.pone.0051396

Roy S, Narayana Y, Balaji KN, Ajitkumar P (2012) Highly fluorescent GFPm 2+-based genome integration-proficient promoter probe vector to study *Mycobacterium tuberculosis* promoters in infected macrophages. Microb Biotechnol 5(1):98–105. doi:10.1111/j.1751-7915.2011.00305.x

Ruth C, Glieder A (2010) Perspectives on synthetic promoters for biocatalysis and biotransformation. Chembiochem 11(6):761–765. doi:10.1002/cbic.200900761

Sala C, Forti F, Di Florio E, Canneva F, Milano A, Riccardi G, Ghisotti D (2003) *Mycobacterium tuberculosis* FurA autoregulates its own expression. J Bacteriol 185(18):5357–5362

Saubi N, Gea-Mallorqui E, Ferrer P, Hurtado C, Sanchez-Ubeda S, Eto Y, Gatell JM, Hanke T, Joseph J (2014) Engineering new mycobacterial vaccine design for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG. Mol Ther Methods Clin Dev 1:14017. doi:10.1038/mtm.2014.17

Schnappinger D, Ehrt S (2014) Regulated expression systems for mycobacteria and their applications. Microbiol Spectr 2(1). doi:10.1128/microbiolspec.MGM2-0018-2013

Seixas FK, Borsuk S, Fagundes MQ, Hartwig DD, Da Silva EF, Cerqueira GM, Dellagostin OA (2010) Stable expression of *Leptospira interrogans* antigens in auxotrophic *Mycobacterium bovis* BCG. Biol Res 43(1):13–18

Seixas FK, da Silva EF, Hartwig DD, Cerqueira GM, Amaral M, Fagundes MQ, Dossa RG, Dellagostin OA (2007) Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. Vaccine 26(1):88–95. doi: 10.1016/j.vaccine.2007.10.052

Shawar RM, Humble DJ, Van Dalfsen JM, Stover CK, Hickey MJ, Steele S, Mitscher LA, Baker W (1997) Rapid screening of natural products for antimycobacterial activity by using luciferase-expressing strains of *Mycobacterium bovis* BCG and *Mycobacterium intracellulare*. Antimicrob Agents Chemother 41(3):570–574

Shi C, Chen L, Chen Z, Zhang Y, Zhou Z, Lu J, Fu R, Wang C, Fang Z, Fan X (2010) Enhanced protection against tuberculosis by vaccination with recombinant BCG over-expressing HspX protein. Vaccine 28(32):5237–5244. doi:10.1016/j.vaccine.2010.05.063

Singh V, Biswas RK, Singh BN (2014) Double recombinant *Mycobacterium bovis* BCG strain for screening of primary and rationale-based antimycobacterial compounds. Antimicrob Agents Chemother 58(3):1389–1396. doi:10.1128/AAC.01301-13

Singh VK, Srivastava R, Srivastava BS (2016) Manipulation of BCG vaccine: a double-edged sword. Eur J Clin Microbiol infect dis 35(4):535–543. doi:10.1007/s10096-016-2579-y

Speranza V, Colone A, Cicconi R, Palmieri G, Giovannini D, Grassi M, Mattei M, Sali M, Delogu G, Andreola F, Colizzi V, Mariani F (2010) Recombinant BCG-Rv1767 amount determines, in vivo, antigen-specific T cells location, frequency, and protective outcome. Microb Pathog 48(5):150–159. doi:10.1016/j.micpath.2010.02.003

Springer B, Sander P, Sedlacek L, Ellrott K, Bottger EC (2001) Instability and site-specific excision of integration-proficient mycobacteriophage L5 plasmids: development of stably maintained integrative vectors. Int J Med Microbiol 290(8):669–675. doi:10.1016/S1438-4221(01)80004-7

Stover CK, Bansal GP, Hanson MS, Burlein JE, Palaszynski SR, Young JF, Koenig S, Young DB, Sadziene A, Barbour AG (1993) Protective immunity elicited by recombinant bacille Calmette- Guerin (BCG) expressing outer surface protein a (OspA) lipopro- tein: a candidate Lyme disease vaccine. J Exp Med 178(1):197–209

Stover CK, de la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, Bansal GP, Young JF, Lee MH, Hatfull GF, Snapper SB, Barletta RG, Jacobs WR Jr, Bloom BR (1991) New use of BCG for recom- binant vaccines. Nature 351(6326):456–460. doi:10.1038/351456a0

Streit JA, Recker TJ, Donelson JE, Wilson ME (2000) BCG expressing LCR1 of *Leishmania chagasi* induces protective immunity in sus- ceptible mice. Exp Parasitol 94(1):33–41. doi:10.1006/expr.1999. 4459S0014-4894(99)94459-4

Sukumar N, Tan S, Aldridge BB, Russell DG (2014) Exploitation of *Mycobacterium tuberculosis* reporter strains to probe the impact of vaccination at sites of infection. PLoS Pathog 10(9):e1004394. doi: 10.1371/journal.ppat.1004394

Sun R, Skeiky YA, Izzo A, Dheenadhayalan V, Imam Z, Penn E, Stagliano K, Haddock S, Mueller S, Fulkerson J, Scanga C, Grover A, Derrick SC, Morris S, Hone DM, Horwitz MA, Kaufmann SH, Sadoff JC (2009) Novel recombinant BCG express- ing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*. Vaccine 27(33):4412–4423. doi:10.1016/j.vaccine.2009.05.048

Timm J, Lim EM, Gicquel B (1994a) *Escherichia coli*-mycobacteria shuttle vectors for operon and gene fusions to lacZ: the pJEM series. J Bacteriol 176(21):6749–6753

Timm J, Perilli MG, Duez C, Trias J, Orefici G, Fattorini L, Amicosante G, Oratore A, Joris B, Frere JM, Pugsley AP, Gicquel B (1994b) Transcription and expression analysis, using lacZ and phoA gene fusions, of *Mycobacterium fortuitum* beta-lactamase genes cloned from a natural isolate and a high-level beta-lactamase producer. Mol Microbiol 12(3):491–504

Triccas JA, Parish T, Britton WJ, Gicquel B (1998) An inducible expres- sion system permitting the efficient purification of a recombinant antigen from *Mycobacterium smegmatis*. FEMS Microbiol Lett 167(2):151–156

Varaldo PB, Leite LC, Dias WO, Miyaji EN, Torres FI, Gebara VC, Armoa GR, Campos AS, Matos DC, Winter N, Gicquel B, Vilar MM, McFadden J, Almeida MS, Tendler M, McIntosh D (2004) Recombinant *Mycobacterium bovis* BCG expressing the Sm14 an- tigen of *Schistosoma mansoni* protects mice from cercarial chal- lenge. Infect Immun 72(6):3336–3343. doi:10.1128/IAI.72.6.3336-3343.200472/6/3336

Via LE, Curcic R, Mudd MH, Dhandayuthapani S, Ulmer RJ, Deretic V (1996) Elements of signal transduction in *Mycobacterium tuberculosis*: in vitro phosphorylation and in vivo expression of the response regulator MtrA. J Bacteriol 178(11):3314–3321

Wiker HG, Harboe M (1992) The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. Microbiol Rev 56(4):648–661

Yuan X, Teng X, Jing Y, Ma J, Tian M, Yu Q, Zhou L, Wang R, Wang W, Li L, Fan X (2015) A live attenuated BCG vaccine overexpressing multistage antigens Ag85B and HspX provides superior protection against *Mycobacterium tuberculosis* infection. Appl Microbiol Biotechnol 99(24):10587–10595. doi:10.1007/s00253-015-6962- x10.1007/s00253-015-6962-x

Yuan Y, Crane DD, Simpson RM, Zhu YQ, Hickey MJ, Sherman DR, Barry CE 3rd (1998) The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. Proc Natl Acad Sci U S A 95(16):9578–9583

Zahrt TC, Deretic V (2000) An essential two-component signal transduc- tion system in *Mycobacterium tuberculosis*. J Bacteriol 182(13): 3832–3838

Zhang T, Li SY, Nuermberger EL (2012) Autoluminescent *Mycobacterium tuberculosis* for rapid, real-time, non-invasive as- sessment of drug and vaccine efficacy. PLoS One 7(1):e29774. doi:10.1371/journal.pone.0029774

Zhu YD, Fennelly G, Miller C, Tarara R, Saxe I, Bloom B, McChesney M (1997) Recombinant bacille Calmette-Guerin expressing the measles virus nucleoprotein protects infant rhesus macaques from measles virus pneumonia. J Infect Dis 176(6):1445–145

3.2 Artigo 2

A standardized BioBrick toolbox for assembly of sequences from mycobacteria

Manuscrito a ser submetido ao periódico *ACS Synthetic Biology*

A standardized BioBrick toolbox for assembly of sequences from mycobacteria

Thaís Larré Oliveira,[1] Carlos Eduardo Pouey da Cunha,[1] Caroline Rizzi,[1] Anna Stedman,[2] Antonio Sergio Varela Junior,[3] Karine Rech Begnini,[1] Johnjoe McFadden,[2] Odir Antônio Dellagostin[1*]

Affiliations:

[1] Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

[2] Department of Microbial and Cellular Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, United Kingdom.

[3] Instituto de Ciências Biológicas, Universidade Federal de Rio Grande, Rio Grande, RS, Brazil.

***Corresponding Author:**

Odir Antônio Dellagostin, Centro de Desenvolvimento Tecnológico – Núcleo de Biotecnologia; Universidade Federal de Pelotas, Campus Universitário, Caixa Postal 354, CEP 96010–900, Pelotas, RS, Brazil. Tel. +55 53 3275 7616; Fax +55 53 3275 7551. Email: odirad@ufpel.edu.br

Abstract

For more than 25 years, recombinant *Mycobacterium bovis* BCG has been genetically engineered for use as a vehicle for antigen expression and immunomodulation. However, BCG efficacy is still unpredictable, and cloning of sequences from mycobacteria is poorly reproducible due to the lack of standardization. To overcome such limitations, we have employed the BioBrick format to construct a toolbox of several mycobacterial parts, including coding sequences, reporter genes, selective markers, promoters, and other regulatory sequences. Additionally, we developed and characterized BioBrick-compatible episomal vectors that are able to replicate in BCG for expression of heterologous antigens. Thus, from now on, any coding sequence cloned in BioBrick format may be expressed in BCG under the control of different promoters, using the vectors constructed in this study. We believe that this mycobacterial toolbox represents a standardized and useful kit for use of recombinant BCG.

Keywords: BCG; BioBrick; toolbox;vector**.**

Introduction

The methods for conventional cloning of DNA are often expensive, laborious, unpredictable, and not reproducible.[1] To overcome these issues, Knight and coworkers described an assembly strategy named "BioBricks" that aims to standardize the construction of basic parts of DNA by the use of prefix and suffix tags flanking the target sequence. This strategy allows fusing different sequences head-to-tail using consecutive and identical digestion and ligation steps.[2] The BioBrick parts are listed in the Registry of Standard Biological Parts and are available for the community[3]; however, most of them were constructed for model organisms, such as *Escherichia coli* and *Bacillus subtilis.*[4-6]

The Registry has over 20,000 standardized biological sequences[7] and, despite several synthetic biology projects having been developed using other assembly strategies, such as Gibson assembly, Golden Gate, or MoClo,[8-10] the majority of these parts are compatible with the BioBrick standard described in the Request for Comments 10 (RFC10). The RFC10 proposes an idempotent design for transcriptional assemblies, ensuring compatibility between parts.[11] However, translational assemblies are not allowed using RFC10 because when two parts are digested with *Xba*I and *Spe*I and their overhangs are ligated, the 6 pb-scar formed creates a frameshift and a stop codon. The Silver RFC23 is compatible with RFC10 and was established to overcome this limitation, allowing in-frame fusion of protein domains.[12]

Mycobacterium bovis Bacillus Calmette–Guerin (BCG) is an attenuated vaccine widely used against tuberculosis (TB) that also offers the potential to be used as a live vaccine vector.[13] BCG is safe, stable, and able to replicate inside antigen-presenting cells as well as to induce strong and long-lasting immune responses.[14] Antigens from several pathogens have been expressed in BCG under the control of different promoters, signal sequences, and other regulatory elements.[15] Moreover, overexpression of mycobacterial antigens in BCG is an alternative that has been explored to improve the efficacy of BCG against TB.[16-17] Recombinant BCG (rBCG) has also been successfully used in cancer immunotherapy.[18]

Despite all these approaches, the cloning of sequences to construct rBCG strains still lacks standardization. It is well known that promoter strength, location of foreign antigens, and the presence or absence of antibiotic resistance genes are determinant factors for the stability and efficacy of rBCG strains.[19] These factors are determined by different DNA sequences, which are frequently shared in a variety of projects with rBCG. Standardization of these sequences would simplify assembly and allow reproducibility in construction of rBCG.

The aim of this study was to standardize the mycobacterial sequences commonly used in the construction of rBCG as BioBrick parts. Furthermore, we created replicative expression plasmids harboring seven different promoters and characterized their functionality in rBCG cultured in vitro and inside macrophages using *egfp* as a reporter gene.

Results and Discussion

BioBrick-compatible mycobacterial parts

All sequences in the mycobacterial toolbox constructed in this study were cloned as BioBrick parts into pSB1C3, the standard plasmid from the BioBricks Repository (Table 1). The fragments containing the restriction sites associated with the BioBrick RFC 10 standard had these sequences removed by commercial synthesis.

The expression of foreign antigens is driven by the use of different promoters, the activity of which determines antigen expression level and also affects the stability of the vaccine vector.[15] Here, we constructed 10 different promoters as BioBrick parts, including three mutagenized promoters generated in a previous study[20] through error-prone PCR of the strong PL5 promoter (PL5X, PL5Y, and PL5Z), five classic (pAN, 18 kDa, Hsp60, Ag85B, and Sag85B), and two inducible promoters (HspX and HspXT). The classic promoters have been extensively used to drive expression of viral, bacterial, and parasitic antigens in BCG.[15] The truncated and whole sequences of the HspX promoter are differentially regulated in virulent or avirulent mycobacteria strains grown in liquid media, low-oxygen conditions, or inside macrophages.[21-22] Promoters pL5X, pL5Y, and pL5Z are mutated versions of the strong promoter pL5, derived from mycobacteriophage L5, and were identified as having low, intermediate, and high strengths, respectively.[20] Here, the strength of classic and inducible promoters, as well as the functionality of these constructs, was further determined through a systematic strategy using the same plasmid backbone and the same reporter gene under control of each promoter.

The coding sequences in our mycobacterial BioBrick toolbox include the mycobacterial sequences *ag85B* and *leuD* and the reporter gene *egfp*. The 85B antigen is part of a complex of proteins that play an important role in mycobacterial pathogenesis[23-24] and, for this reason, it has been extensively evaluated as a vaccine candidate to improve BCG efficacy.[25-26] The *leuD* gene codes for an essential enzyme in leucine biosynthesis and has been used in auxotrophic complementation systems[27] to obtain more stable and safer rBCG vaccine strains.[28-29] The sequence of mycobacterial origin of replication, commercially synthesized, and the integrase, derived from mycobacteriophage L5,[30] were cloned into pSB1C3 to allow the construction of both episomal and integrative vectors to work with mycobacteria. Genome integration or the maintenance of the vector episomally represent genetic systems used for the expression of heterologous genes in BCG, with effects in strain stability and antigen expression levels.[31] Also, the resistance gene to kanamycin was cloned as a BioBrick part, and both the origin of replication and their functionality was demonstrated by visualization of colony growth in selective media with kanamycin.

Construction of new expression vectors for mycobacteria

Aiming to allow the expression of heterologous antigens in rBCG according to BioBrick format, we constructed a base plasmid named pUP500 able to replicate in *E. coli* and *M. bovis* BCG containing a multiple cloning site with the prefix and suffix BioBrick-sequences (Figure 1). The pUP500 plasmid contains a resistance gene to kanamycin flanked by restriction sites for *Hind*III. This strategy allows the deletion of the antibiotic selective marker for construction of auxotrophic BCG strains using the same plasmid backbone. Recent work of our group has shown that a leucine auxotroph expressing Ag85B is a promising alternative to improve BCG efficacy against tuberculosis, generating more stability in vitro and in vivo compared to the BCG Pasteur strain.[28]. The use of auxotrophic strains has been explored to maintain selective pressure in vivo and improve vaccine safety.[27]

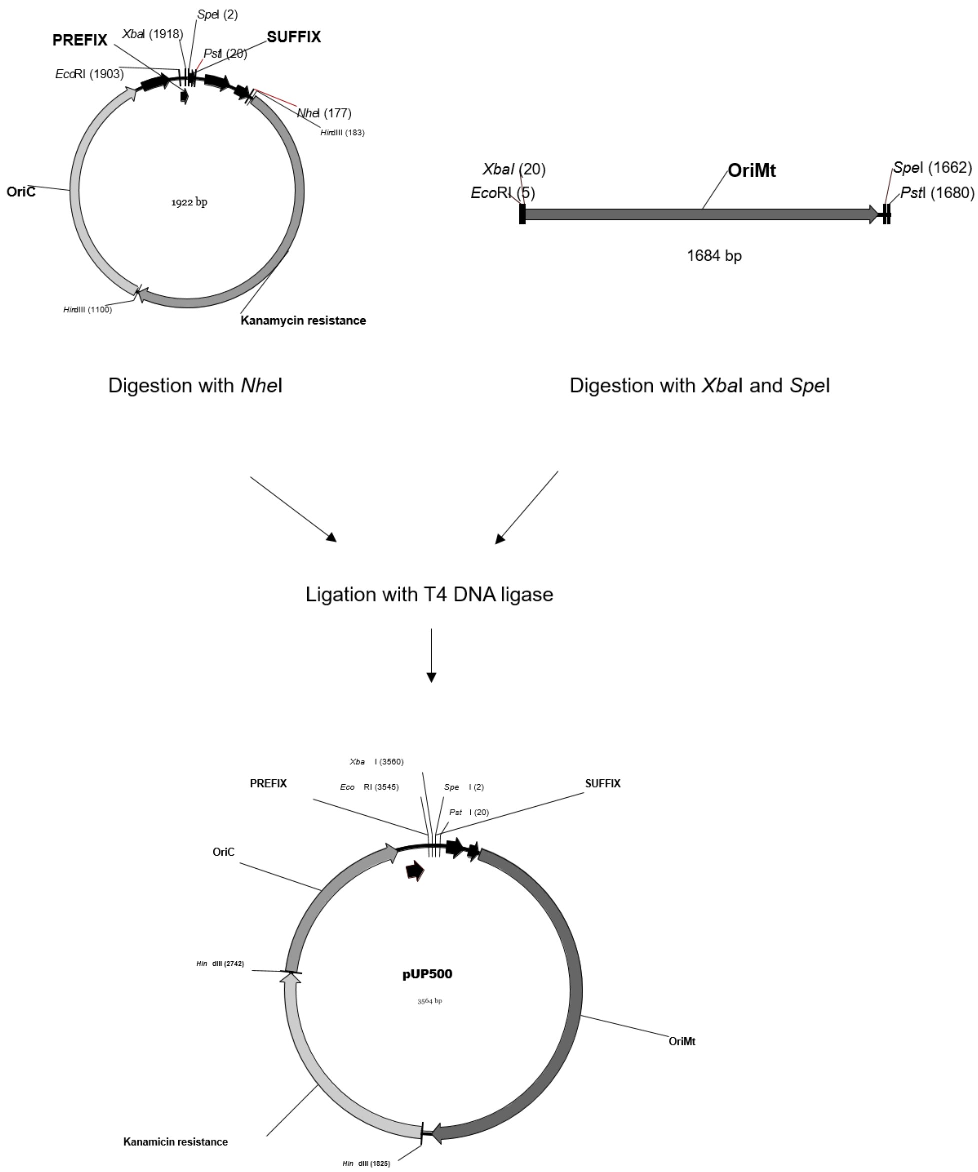


Figure 1. Schematic outline of the construction of the base vector pUP500. The sequence of origin of replication cloned into the vector pUP500 was commercially synthesized. The vector was digested with the enzyme *Nhe*I and the sequence of the origin of replication was digested with *Xba*I and *Spe*I; after, the two molecules were ligated using the enzyme T4 DNA ligase and the functionality of the constructed pUP500 vector was confirmed for its ability to replicate in BCG.

Seven different promoter sequences were individually cloned in pUP500, generating pUP500/P_{pAN}, pUP500/P_{18kDa}, pUP500/P_{hsp60}, pUP500/P_{ag85B}, pUP500/P_{sag85B}, pUP500/P_{hspX}, and pUP500/P_{hspXT} (Figure 2). Structural and functional characteristics of the plasmid may affect copy number and vaccine stability due to the metabolic burden associated with maintaining it in the bacterial cell.[19] The use of a common plasmid backbone benefits the standardization and reproducibility of rBCG strains obtained and decreases both the bias of plasmid copy number in antigen expression variations and the unpredictability of vaccine efficacy. These vectors are available for expression of any antigens cloned according to the BioBrick format in BCG.

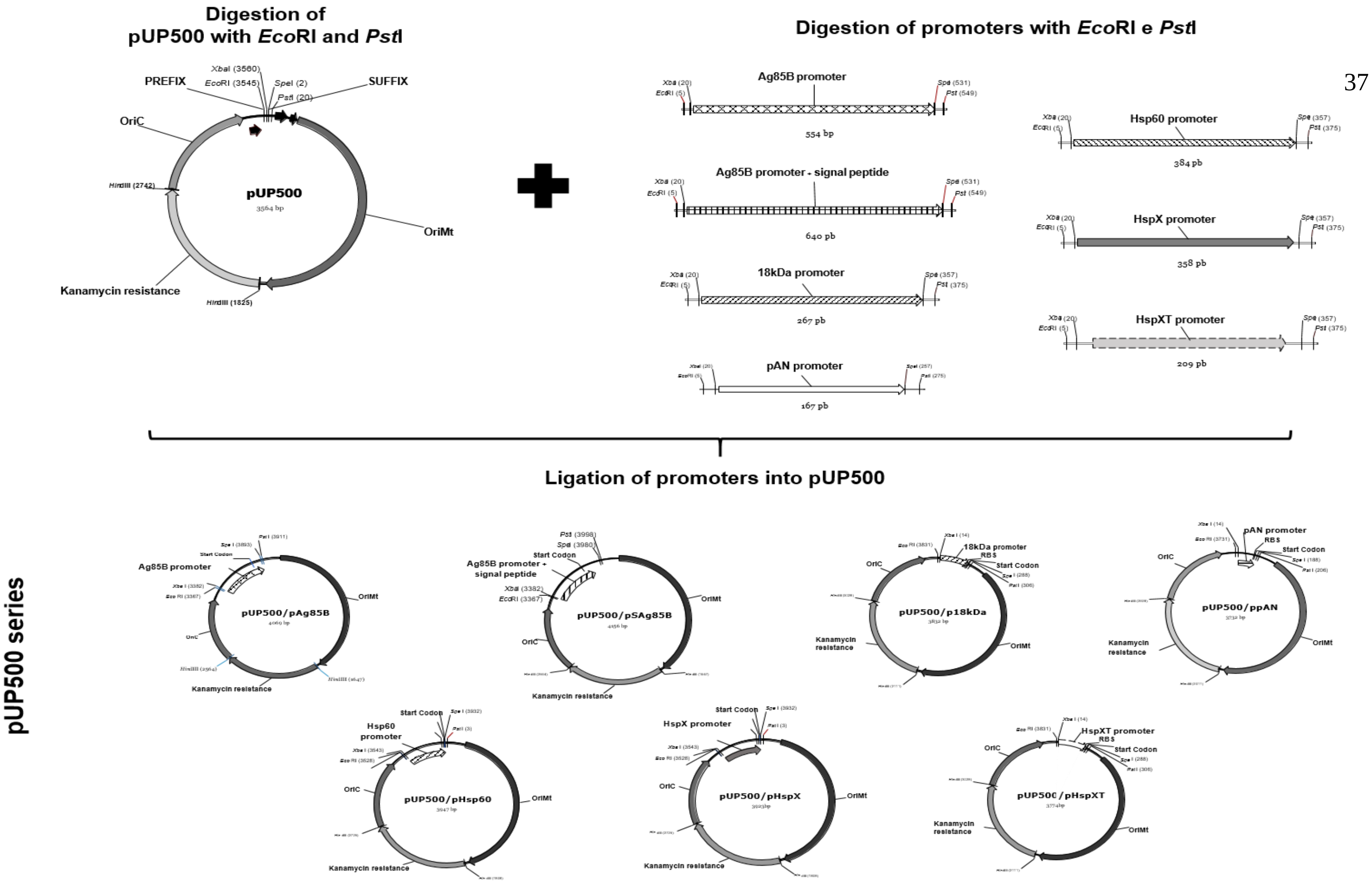


Figure 2. Schematic outline of the pUP500 series of vectors constructed in this study. The pUP500 vector and the sequences of the mycobacterial promoters were digested with the *Eco*RI and *Pst*I enzymes; after, promoters were individually ligated into pUP500 using the enzyme T4 DNA ligase, generating the pUP500 series that consists of pUP500/P_{pAN}, pUP500/P_{18kDa}, pUP500/P_{hsp60}, pUP500/P_{ag85B}, pUP500/P_{sag85B}, pUP500/P_{hspX} and pUP500/P_{hspXT}.

Characterization of mycobacterial promoters using *egfp*

In this study, we used the eGFP as a reporter protein to determine whether our constructs were functional and to assess the activity of different promoters in rBCG cultured in vitro and inside macrophages. This protein has mutations in two amino acids that result in higher levels of fluorescence than the wild-type GFP from jellyfish *Aequeora victoria.*[32-33] The expression of eGFPwas driven by the upstream promoter sequences of the pUP500 series of vectors and determined by fluorescence microscopy and flow cytometry (Figure 3). Kanno and colleagues demonstrated that measurement of fluorescence levels of eGFP by flow cytometry is a reliable tool to determine promoter activity, and it correlates with total amount of this protein in SDS-PAGE.[20]

In comparison to the wild-type BCG Pasteur, a higher percentage of fluorescent BCG cells was observed for constructs using pAN and 18 kDa promoters, followed by HspX, the truncated sequence of HspX (HspXT), and Ag85B ($P< 0.05$). No difference was observed when Hsp60 or Ag85B plus signal sequence were used to drive expression of eGFP (Figure 3C). When the intensity of fluorescence was determined for gated eGFP-positive BCG cells (Figure 3D), these differences were maintained, except for the HspXT that did not differ from the control in this analysis ($P > 0.05$).

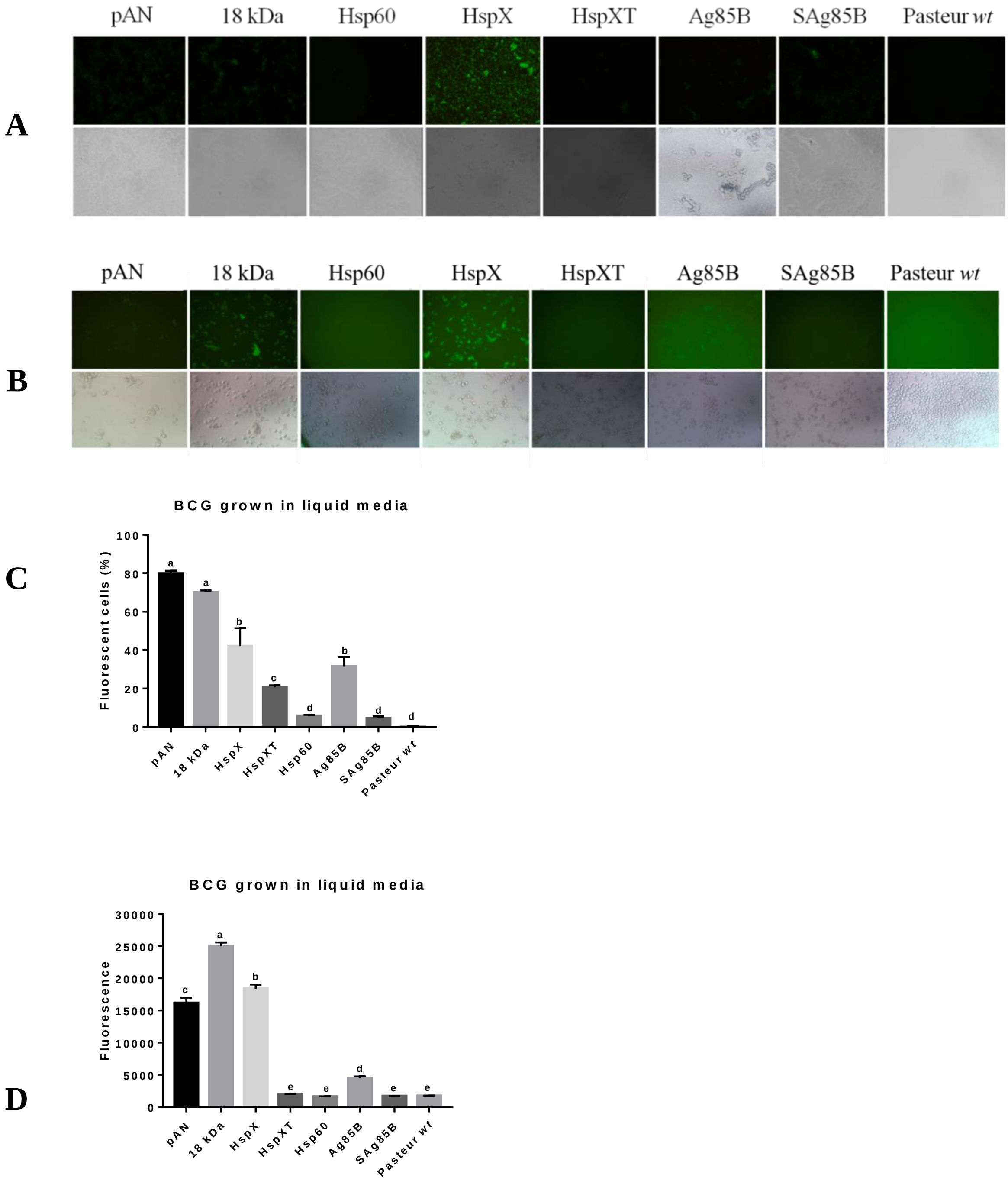


Figure 3. Fluorescence detection induced by pUP500 plasmids in rBCG cultured in vitro and inside macrophages. The expression of *egfp* was driven by the upstream promoter sequences of the pUP500 series of vectors, as indicated, and confirmed by fluorescence microscopy (A and B) and flow cytometry (C and D). Wild-type BCG Pasteur was used as negative control in both growth conditions. Expression of eGFP detected by fluorescence microscopy in rBCG harboring pUP500 plasmids cultured in vitro (**A**) or inside macrophages (**B**); **C**, Percentage of BCG cells grown in liquid media expressing eGFP under control of different promoters; **D**, Intensity of fluorescence in gated-positive eGFP BCG cells grown in liquid media. Statistical analysis was performed by one-way ANOVA; different letters indicate significant differences (*P* <0.05) between samples.

The Hsp60 is considered to be a strong promoter that is widely used for expression of heterologous antigens in rBCG.[15, 19] However, here we have demonstrated that no significant eGFP expression was detected when this promoter was used. Kanno and colleagues also observed weak expression levels of GFP in *M. smegmatis* using the Hsp60 promoter, even when cells were exposed to heat shock treatment[20]; this may be attributed to a high metabolic load imposed by eGFP synthesis controlled by this promoter. Other studies have reported structural instability of Hsp60, which can be associated with failure of a vectorized-BCG vaccine against leptospirosis.[34-35] The constructions pUP500/P_{hsp60} and pUP500/P_{sag85B} were used to express antigens from *Leptospira interrogans* in BCG successfully, demonstrating their functionality (data not published).

When the HspX and HspXT promoter sequences were evaluated, we observed higher levels of eGFP expression driven by full sequence of the HspX promoter (P <0.05), in comparison with the truncated one (HspXT). Dokladda and colleagues also observed that HspX is induced in avirulent strains both in vitro and inside macrophages, while the HspXT promoter was activated only in virulent strains grown into macrophages or under conditions of low oxygen tension.[21]

We also demonstrated that, inside macrophages, rBCG strains harboring pUP500 plasmids expressed eGFP similarly to that observed in vitro (Figure 3B). No expression was detected in macrophages infected with *wt*BCG.By fluorescence microscopy, low fluorescence intensity was observed in macrophages infected with strains in which the promoters Hsp60, HspXT, and Sag85B were used to drive eGFP expression. Fluorescence levels seem to have been higher in macrophages than in vitro when the promoter 18 kDa was used. This is expected due to activation of this promoter during intracellular growth.[36] However, a quantitative analysis must be performed to confirm these results. Moreover, other mycobacterial hosts, such as *M. smegmatis*, should be used to evaluate the functionality of vectors constructed in this study and the reproducibility of our results.

Methods

Bacterial strains and growth conditions

Escherichia coli strain DH5α (Invitrogen) was cultured in Luria–Bertani medium at 37°C with the addition of kanamycin or chloramphenicol (Sigma-Aldrich, Missouri, United States) to 50 $\mu g.ml^{-1}$, when required. *Mycobacterium bovis* BCG Pasteur was grown at 37ºC in Middlebrook 7H9 medium (Difco) containing 10% of oleic acid, albumin, dextrose complex (OADC – Difco, New Jersey, United States), 0.2% glycerol, and 0.05% Tween 80 (Sigma-Aldrich, Missouri, United States); or 7H10 agar (Difco, New Jersey, United States) supplemented with 10% OADC and 0.2% glycerol, with or without kanamycin (25 $\mu g.ml^{-1}$).

Reagents and DNA manipulation

Oligonucleotides were synthesized by Exxtend (Sao Paulo, Brazil). PCR reactions were performed using GoTaq® Colorless Master Mix (Promega, Wisconsin, United States). Restriction enzymes and T4 DNA ligase enzyme were purchased from New England Biolabs (Massachusetts, United States). Purification of PCR and digestion products was performed with the *GFX™ PCR and Gel Band Purification Kit* (GE Healthcare, Illinois, United States). The plasmid pSB1C3 was acquired from the Registry of Standard Biological Parts. Plasmid preparation was performed with *GFX™ Micro Plasmid PrepKit* (GE Healthcare, Illinois, United States).All primer sequences and plasmids constructed in this study are described in Tables 1 and 2, respectively.

Construction of standard basic parts

The promoters P_{pAN}, P_{18kDa}, and P_{hsp60} were amplified, respectively, from the following plasmids previously constructed: pUS977, pUS2000, and pUS973[34, 37] (Table 1). The promoters P_{ag85B}, P_{sag85B}, P_{hspX} and its truncated sequence (P_{hspXT}) were obtained from genomic DNA of *M. bovis* BCG Pasteur. The promoters PL5X, PL5Y, and PL5Z were generated by error-prone PCR of the PL5 promoter in a previous work.[20] The *leuD* sequencewas obtained from pUP410.[27]The *ag85b* coding sequence and the mycobacterial origin of replication were commercially synthesized to remove BioBrick restriction sites in their sequences and then amplified by PCR. The integrase cassette, derived from mycobacteriophage L5, was obtained from the plasmid pMV306 and refers to the gene encoding the phage integrase protein and the *attP* site.[38] All fragments were cut with *Eco*RI and *Pst*I, ligated into pSB1C3 (BioBrick submission vector), and confirmed by sequencing. A BioBrick replicative vector for expression in BCG, named pUP500, was commercially synthesized with the *E. coli* origin of replication, a resistance gene to kanamycin flanked by *Hind*III restriction sites and the BioBrick multiple cloning site containing the prefix and suffix sequences. Moreover, pUP500 has a *Nhe*I restriction site where the mycobacterial origin of replication was inserted. Briefly, the mycobacterial origin of replication was amplified by PCR, digested with *Xba*I and *Spe*I, and ligated into pUP500 previously digested with *Nhe*I. Afterwards, *E. coli* DH5α competent cells were electroporated and plated on selective medium. Recombinant clones were confirmed by PCR, digestion, and sequencing. To access the functionality of pUP500 for replication in BCG, competent cells of BCG Pasteur were electroporated and plated on selective medium.

Assembly of BioBrick vectors for expression in rBCG

Promoter sequences were amplified by PCR, digested with *Eco*RI and *Pst*I, and cloned into pUP500 digested with the same enzymes. The resulting constructions pUP500/promoters were then digested with *Spe*I and *Pst*I and ligated with the reporter gene *egfp*, amplified from pRSET-EmGFP (Invitrogen), and digested with *Xba*I and *Pst*I. *E. coli* DH5α competent cells were electroporated with the recombinant plasmids, and the cloning of *egfp* downstream of each promoter into pUP500 was confirmed by sequencing.

BCG transformation and analysis of expression

Electrocompetent cells of *M. bovis* BCG Pasteur were transformed with the plasmids pUP500/PpAN:*egfp*, pUP500/P18kDa:*egfp*, pUP500/Phsp60:*egfp*, pUP500/Pag85B:*egfp*, pUP500/Psag85B:*egfp*, pUP500/PhspX:*egfp*,and pUP500/PhspXT:*egfp*, and recombinant strains were selected in 7H10 medium with kanamycin. Recombinant BCG strains were grown for 5 days in selective 7H9 medium and visualized with a fluorescence microscope at 20× magnification.

Macrophage infection

The J774.A1 cell line was cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Scientific, Illinois, United States) supplemented with 10% (v/v) fetal bovine serum in a concentration of 5 x 10^4 cells/well. Macrophage infection was performed for 2 h using mycobacterial cultures with an optical density at 600 nm of 0.6 in a multiplicity of infection (MOI) of 1:500. Bacteria that were not internalized were removed after three washes with PBS, and the infected cells were maintained in culture for 24 h. Subsequently, infected macrophages were washed with PBS and visualized under inverted fluorescence microscopy.

Characterization of eGFP expression in rBCG strains

AnAttune® Acoustic Focusing Cytometer, Blue/Violet (Applied Biosystems, California, United States) was used to determine the single-cell fluorescence of gated bacterial cells on the basis of the median of eGFP (with 488 nm excitation and 509 nm emission filters). A total of 10,000 events per sample were collected. Expression of eGFP was evaluated in terms of percentage of fluorescent cells and intensity of fluorescence among positive cells.

Statistical analysis

The fluorescence levels were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-test for multiple comparisons. Significance was set at P value < 0.05. All data were expressed as mean ± SEM.

Conclusions

Our genetic toolbox contains 10 different promoters, 2 mycobacterial coding sequences, a reporter gene, an antibiotic resistance gene, and a BioBrick-compatible replicative vector. The functionality of these parts was demonstrated by flow cytometry and fluorescence microscopy, and the strength of promoters was evaluated in a systematic strategy. The technology we have applied to assemble sequences from mycobacteria will support other projects with recombinant BCG. Moreover, we provide a range of vectors for expression of foreign antigens in BCG. We believe that our study will allow the elucidation of the influence of molecular characteristics in the response induced by BCG-based vaccines in a more economical and practical manner than conventional cloning, as well as demonstrating the use of this kit applied to other pathogens.

Acknowledgements

We are grateful to Michele dos Santos for the technical assistance provided during this study. This work was funded by grants from CNPq, CAPES and FAPERGS, Brazilian Research Funding Agencies.

References

(1) Muller, K. M., and Arndt, K. M. (2012) Standardization in synthetic biology. *Methods Mol Biol. 813*, 23-43.

(2) Shetty, R. P., Endy, D., and Knight, T. F., Jr. (2008) Engineering BioBrick vectors from BioBrick parts. *J Biol Eng. 2*, 5.

(3) Smolke, C. D. (2009) Building outside of the box: iGEM and the BioBricks Foundation. *Nat Biotechnol. 27* (12), 1099-102.

(4) Radeck, J., Kraft, K., Bartels, J., Cikovic, T., Durr, F., Emenegger, J., Kelterborn, S., Sauer, C., Fritz, G., Gebhard, S., and Mascher, T. (2013) The *Bacillus* BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with Bacillus subtilis. *J Biol Eng. 7* (1), 29.

(5) Levskaya, A., Chevalier, A. A., Tabor, J. J., Simpson, Z. B., Lavery, L. A., Levy, M.,

Davidson, E. A., Scouras, A., Ellington, A. D., Marcotte, E. M., and Voigt, C. A. (2005) Synthetic biology: engineering *Escherichia coli* to see light. *Nature. 438* (7067), 441-2.

(6) Popp, P. F., Dotzler, M., Radeck, J., Bartels, J., and Mascher, T. (2017) The *Bacillus* BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with *Bacillus subtilis*. *Sci Rep. 7* (1), 15058.

(7) Registry of Standard Biological Parts. http://parts.igem.org/Main_Page

(8) Gibson, D. G. (2009) Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. *Nucleic Acids Res. 37* (20), 6984-90.

(9) Engler, C., Gruetzner, R., Kandzia, R., and Marillonnet, S. (2009) Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. *PLoS One. 4* (5), e5553.

(10) Werner, S., Engler, C., Weber, E., Gruetzner, R., and Marillonnet, S. (2012) Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system. *Bioeng Bugs. 3* (1), 38-43.

(11) Registry of Standard Biological Parts RFC10 standard. http://parts.igem.org/Help:Standards/Assembly/RFC10

(12) Registry of Standard Biological Parts RFC23 standard. http://parts.igem.org/Help:Standards/Assembly/RFC23 (2013).

(13) Singh, V. K., Srivastava, R., and Srivastava, B. S. (2016) Manipulation of BCG vaccine: a double-edged sword. *Eur J Clin Microbiol Infect Dis. 35* (4), 535-43.

(14) Nasser Eddine, A., and Kaufmann, S. H. (2005) Improved protection by recombinant BCG. *Microbes Infect. 7* (5-6), 939-46.

(15) Bastos, R. G., Borsuk, S., Seixas, F. K., and Dellagostin, O. A. (2009) Recombinant *Mycobacterium bovis* BCG. *Vaccine. 27* (47), 6495-503.

(16) da Costa, A. C., Costa-Junior Ade, O., de Oliveira, F. M., Nogueira, S. V., Rosa, J. D., Resende, D. P., Kipnis, A., and Junqueira-Kipnis, A. P. (2014) A new recombinant BCG vaccine induces specific Th17 and Th1 effector cells with higher protective efficacy against tuberculosis. *PLoS One. 9* (11), e112848.

(17) da Costa, A. C., Nogueira, S. V., Kipnis, A., and Junqueira-Kipnis, A. P. (2014) Recombinant BCG: Innovations on an Old Vaccine. Scope of BCG Strains and Strategies to Improve Long-Lasting Memory. *Front Immunol. 5*, 152.

(18) Fuge, O., Vasdev, N., Allchorne, P., and Green, J. S. (2015) Immunotherapy for bladder cancer. *Res Rep Urol. 7*, 65-79.

(19) Oliveira, T. L., Rizzi, C., and Dellagostin, O. A. (2017) Recombinant BCG vaccines:

molecular features and their influence in the expression of foreign genes. *Appl Microbiol Biotechnol.*

(20) Kanno, A. I., Goulart, C., Rofatto, H. K., Oliveira, S. C., Leite, L. C., and McFadden, J. (2016) New Recombinant *Mycobacterium bovis* BCG Expression Vectors: Improving Genetic Control over Mycobacterial Promoters. *Appl Environ Microbiol. 82* (8), 2240-6.

(21) Dokladda, K., Billamas, P., and Palittapongarnpim, P. (2015) Different behaviours of promoters in *Mycobacterium tuberculosis* H37Rv and H37Ra. *World J Microbiol Biotechnol. 31* (2), 407-13.

(22) Yuan, Y., Crane, D. D., Simpson, R. M., Zhu, Y. Q., Hickey, M. J., Sherman, D. R., and Barry, C. E., 3rd. (1998) The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc Natl Acad Sci U S A. 95* (16), 9578-83.

(23) Wiker, H. G., and Harboe, M. (1992) The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiol Rev. 56* (4), 648-61.

(24) Belisle, J. T., Vissa, V. D., Sievert, T., Takayama, K., Brennan, P. J., and Besra, G. S. (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science. 276* (5317), 1420-2.

(25) Rizzi, C., Bianco, M. V., Blanco, F. C., Soria, M., Gravisaco, M. J., Montenegro, V., Vagnoni, L., Buddle, B., Garbaccio, S., Delgado, F., Leal, K. S., Cataldi, A. A., Dellagostin, O. A., and Bigi, F. (2012) Vaccination with a BCG strain overexpressing Ag85B protects cattle against *Mycobacterium bovis* challenge. *PLoS One. 7* (12), e51396.

(26) Horwitz, M. A., Harth, G., Dillon, B. J., and Maslesa-Galic, S. (2006) A novel live recombinant mycobacterial vaccine against bovine tuberculosis more potent than BCG. *Vaccine. 24* (10), 1593-600.

(27) Borsuk, S., Mendum, T. A., Fagundes, M. Q., Michelon, M., Cunha, C. W., McFadden, J., and Dellagostin, O. A. (2007) Auxotrophic complementation as a selectable marker for stable expression of foreign antigens in *Mycobacterium bovis* BCG. *Tuberculosis (Edinb). 87* (6), 474-80.

(28) Rizzi, C., Peiter, A. C., Oliveira, T. L., Seixas, A. C. N., Leal, K. S., Hartwig, D. D., Seixas, F. K., Borsuk, S., and Dellagostin, O. A. (2017) Stable expression of *Mycobacterium bovis* antigen 85B in auxotrophic *M. bovis* bacillus Calmette-Guerin. *Mem Inst Oswaldo Cruz. 112* (2), 123-130.

(29) Hart, B. E., Asrican, R., Lim, S. Y., Sixsmith, J. D., Lukose, R., Souther, S. J., Rayasam, S. D., Saelens, J. W., Chen, C. J., Seay, S. A., Berney-Meyer, L., Magtanong, L.,

Vermeul, K., Pajanirassa, P., Jimenez, A. E., Ng, T. W., Tobin, D. M., Porcelli, S. A., Larsen, M. H., Schmitz, J. E., Haynes, B. F., Jacobs, W. R., Jr., Lee, S., and Frothingham, R. (2015) Stable Expression of Lentiviral Antigens by Quality-Controlled Recombinant *Mycobacterium bovis* BCG Vectors. *Clin Vaccine Immunol. 22* (7), 726-41.

(30) Lee, M. H., Pascopella, L., Jacobs, W. R., Jr., and Hatfull, G. F. (1991) Site-specific integration of mycobacteriophage L5: integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and bacille Calmette-Guerin. *Proc Natl Acad Sci U S A. 88* (8), 3111-5.

(31) Oliveira, T. L., Rizzi, C., and Dellagostin, O. A. (2017) Recombinant BCG vaccines: molecular features and their influence in the expression of foreign genes. *Appl Microbiol Biotechnol. 101* (18), 6865-6877.

(32) Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene. 173* (1 Spec No), 33-8.

(33) Yang, T. T., Cheng, L., and Kain, S. R. (1996) Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res. 24* (22), 4592-3.

(34) Al-Zarouni, M., and Dale, J. W. (2002) Expression of foreign genes in *Mycobacterium bovis* BCG strains using different promoters reveals instability of the hsp60 promoter for expression of foreign genes in *Mycobacterium bovis* BCG strains. *Tuberculosis (Edinb). 82* (6), 283-91.

(35) Seixas, F. K., da Silva, E. F., Hartwig, D. D., Cerqueira, G. M., Amaral, M., Fagundes, M. Q., Dossa, R. G., and Dellagostin, O. A. (2007) Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. *Vaccine. 26* (1), 88-95.

(36) Dellagostin, O. A., Esposito, G., Eales, L. J., Dale, J. W., and McFadden, J. (1995) Activity of mycobacterial promoters during intracellular and extracellular growth. *Microbiology. 141 (Pt 8)*, 1785-92.

(37) Medeiros, M. A., Dellagostin, O. A., Armoa, G. R., Degrave, W. M., De Mendonca-Lima, L., Lopes, M. Q., Costa, J. F., McFadden, J., and McIntosh, D. (2002) Comparative evaluation of *Mycobacterium vaccae* as a surrogate cloning host for use in the study of mycobacterial genetics. *Microbiology. 148* (Pt 7), 1999-2009.

(38) Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H., Hatfull, G. F., and et al. (1991) New use of BCG for recombinant vaccines. *Nature. 351* (6326), 456-60.

Table 1. BioBrick parts of the mycobacterial toolbox and primers used in this study.

BioBrick part	Primers (5'-3')	Reference of source
P_{paN}	F: CCGGAATTCGCGGCCGCTTCTAGACCAAGGCCGAAGAGCCC R: TGCACTGCAGCGGCCGCTACTAGTATCCCTTGACAACGTCAT	**pUS977** [37]
P_{hsp60}	F: CCGGAATTCGCGGCCGCTTCTAGAGACCACAACGACGCGCCC R: TGCACTGCAGCGGCCGCTACTAGTCGTCTTGGCCATTGCGAA	**pUS973** [37]
P_{18kDa}	F: CGGAATTCGCGGCCGCTTCTAGAGGTACCGCAGCGACG R: TGCACTGCAGCGGCCGCTACTAGTCATCAGCATGTGTGGTC	**pUS2000** [34]
P_{ag85B}	F: CCGGAATTCGCGGCCGCTTCTAGACGCTATGTAGCTCCA R: TGCACTGCAGCGGCCGCTACTAGTACCTGTGCCCCTTTG	**gDNA from *M. bovis* BCG Pasteur**
P_{sag85B}	F: CCGGAATTCGCGGCCGCTTCTAGACGCTATGTAGCTCCA R: TGCACTGCAGCGGCCGCTACTAGTCACCAGGCCCGGAAG	**gDNA from *M. bovis* BCG Pasteur**
P_{hspX}	F: CCGGAATTCGCGGCCGCTTCTAGACGCGTAAAGCACCCGATCCTT R: TGCACTGCAGGCGGCCGCTACTAGTTGGTGGCCATTTGATGCCTCCT	**gDNA from *M. bovis* BCG Pasteur**
P_{hspXT}	F: CCGGAATTCGCGGCCGCTTCTAGAGCATGATCAACCTCCGCTGTTC R: TGCACTGCAGGCGGCCGCTACTAGTTGGTGGCCATTTGATGCCTCCT	**gDNA from *M. bovis*BCG Pasteur**

PL5X	F: GTTTCTTCGAATTCGCGGCCGCTTCTAGAGACAGCTATGACCATGATTACG R: GTTTCTTCCTGCAGCGGCCGCTACTAGTAATGCGATCTCCCTTTCC	**pL5 promoter** [20]
PL5Y*	Not applicable	**pL5 promoter** [20]
PL5Z*	Not applicable	**pL5 promoter** [20]
leuD	F: CCGGAATTCGCGGCCGCTTCTAGAATGGAAGCCTTTCAC R: TGCACTGCAGCGGCCGCTACTAGTTTATTATCAGGGGGCGGGTAGA	**pUP410** [27]
Ag85B*	Not applicable	**Commercial synthesis**
OriMt	F: CCGGAATTCGCGGCCGCTTCTAGACCCGACACCCGCTCCC R: TGCACTGCAGCGGCCGCTACTAGTCCAGCCCACCAGCTC	**Commercial synthesis**
int	F: GTTTCTTCGAATTCGCGGCCGCTTCTAGAGGCAACTCCCGGTGCAAC R: GTTTCTTCCTGCAGCGGCCGCTACTAGTATGGCTCATAACACCCCTTG	**pMV306** [38]
kan ^{r}	F: CCGGAATTCGCGGCCGCTTCTAGAGATCAGTACTTTGTGT R: TGCACTGCAGCGGCCGCTACTAGTTTAGAAAAACTCATCG	**pUS977** [37]
egfp	F: CCCGAATTCGCGGCCGCTTCTAGAATGTGAGGAGATTCTCAATGACG R: GCACTGCAGCGGCCGCTACTAGTTCACTTGTAC	**pRSET-EmGFP (Invitrogen™)**

* The coding sequence *Ag85B* and the promoters pL5X and pL5Z contained the prefix and suffix sequences from BioBrick format and were obtained by digestion with *Eco*RIand *Pst*I.

Table 2. Plasmids constructed in this study.

Plasmids	Description
pSB1C3/P$_{paN}$	BioBrick submission vector & mycobacterial pAN promoter
pSB1C3/P$_{hsp60}$	BioBrick submission vector & mycobacterial Hsp60 promoter
pSB1C3/P$_{18kDa}$	BioBrick submission vector & mycobacterial 18 kDa promoter
pSB1C3/P$_{ag85B}$	BioBrick submission vector & mycobacterial Ag85B promoter
pSB1C3/P$_{sag85B}$	BioBrick submission vector & mycobacterial Ag85B promoter plus signal sequence
pSB1C3/P$_{hspX}$	BioBrick submission vector & mycobacterial HspX promoter
pSB1C3/P$_{hspXT}$	BioBrick submission vector & mycobacterial truncated HspX promoter
pSB1C3/PL5X	BioBrick submission vector & mycobacterial pL5X promoter
pSB1C3/PL5Y	BioBrick submission vector & mycobacterial pL5Y promoter
pSB1C3/PL5Z	BioBrick submission vector & mycobacterial pL5Z promoter
pSB1C3/*oriMt*	BioBrick submission vector & mycobacterial origin of replication
pSB1C3/*int*	BioBrick submission vector & mycobacterial integrase
pSB1C3/*kan*	BioBrick submission vector & resistance gene to kanamycin

pSB1C3/*ag85B*	BioBrick submission vector & mycbacterial *ag85B* gene
pSB1C3/*leuD*	BioBrick submission vector & mycobacterial *leuD* gene
pUP500	BCG replicative and BioBrick-compatible vector, kan^r
pUP500/P_{paN}	BCG replicative and BioBrick-compatible vector & mycobacterial pAN promoter
pUP500/ P_{hsp60}	BCG replicative and BioBrick-compatible vector & mycobacterial Hsp60 promoter
pUP500/ P_{18kDa}	BCG replicative and BioBrick-compatible vector & mycobacterial 18 kDa promoter
pUP500/ P_{ag85B}	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter
pUP500/ P_{sag85B}	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter plus signal sequence
pUP500/ P_{hspX}	BCG replicative and BioBrick-compatible vector & mycobacterial HspX promoter
pUP500/ P_{hspXT}	BCG replicative and BioBrick-compatible vector & mycobacterial truncated HspX promoter
pUP500/ $P_{paN:egfp}$	BCG replicative and BioBrick-compatible vector & mycobacterial pAN promoter upstream of *egfp* gene
pUP500/ $P_{hsp60:egfp}$	BCG replicative and BioBrick-compatible vector & mycobacterial Hsp60 promoter upstream of *egfp* gene
pUP500/ $P_{18kDa:egfp}$	BCG replicative and BioBrick-compatible vector & mycobacterial 18 kDa promoter upstream of *egfp* gene
pUP500/ $P_{ag85B:egfp}$	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter upstream of *egfp* gene

pUP500/ P$_{\mathbf{sag85B:}\boldsymbol{egfp}}$	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter plus signal sequence upstream of *egfp* gene
pUP500/ P$_{\mathbf{hspX:}\boldsymbol{egfp}}$	BCG replicative and BioBrick-compatible vector & mycobacterial HspX promoter upstream of *egfp* gene
pUP500/ P$_{\mathbf{hspXT:}\boldsymbol{egfp}}$	BCG replicative and BioBrick-compatible vector & mycobacterial truncated HspX promoter upstream of *egfp* gene

3.3 Artigo 3

Recombinant BCG strains expressing chimeric proteins derived from *Leptospira* protect hamsters against leptospirosis

Manuscrito a ser submetido ao periódico *Vaccine*

Recombinant BCG strains expressing chimeric proteins derived from *Leptospira* protect hamsters against leptospirosis

Thaís Larré Oliveira,[1] Carlos Eduardo Pouey da Cunha,[1] Caroline Rizzi,[1] Jessica Dorneles,[1] Amilton Clair Pinto Seixas Neto,[1] Marta Amaral,[1] Odir Antônio Dellagostin.[1]*

Affiliations:

[1] Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

***Corresponding Author:**

Odir Antônio Dellagostin, Centro de Desenvolvimento Tecnológico – Núcleo de Biotecnologia; Universidade Federal de Pelotas, Campus Universitário, Caixa Postal 354, CEP 96010–900, Pelotas, RS, Brazil. Tel. +55 53 3275 7616; Fax +55 53 3275 7551. Email: odirad@ufpel.edu.br

Abstract

Leptospirosis is a spirochaetal zoonosis that is responsible for one million human cases per year. Fusing multiple immunogenic antigens represents a promising approach to delivering an effective vaccine against leptospirosis. *Mycobacterium bovis* BCG is a potential vaccine vector due to its adjuvant properties and safety. Two chimeric genes based on sequences of *ligANI*, *ligBrep*, *lipL32*, and *lemA*, were individually cloned into five BioBrick vectors with different promoters for antigen expression in BCG. Groups of ten hamsters were vaccinated with rBCG strains in two doses of 10^6 CFU and challenged with 5 x LD_{50} of *L. interrogans* serovar Copenhageni. All rBCG vaccines expressing chimera 1, based on antigens LipL32, LigANI, and LemA protected the hamsters from challenge and renal carrier status (80-100% of survival); for chimera 2, the only vaccine that afforded protection was the vaccine that incorporated the pAN promoter (60% of survival). A single vaccine dose was necessary to induce significant IgG levels; however, humoral response was not related to protection. These findings suggest that the combination of potential vaccine candidates in chimeric antigens and the use of BCG as a live vector are promising strategies by which it is possible to obtain an effective and sterilizing vaccine against leptospirosis.

Keywords: leptospirosis; vaccine; chimera; BCG.

1. Introduction

Leptospirosis is an emerging spirochaetal zoonosis that is distributed throughout the world [1]. The infection, which is caused by pathogenic bacteria from *Leptospira* genus, occurs through contact with the urine of animal reservoirs that harbor the spirochete in renal tubules [2]. Thus, the disease has been associated with occupational risks, such as veterinary medicine and farming, and with recreational activities such as water sports [3]. Moreover, environmental exposures arising from poor sanitation, poverty, and uncollected refuse, are by far the most significant risk factors in urban slum settings such as those commonly observed in developing countries like Brazil [4-5]. Clinical manifestations in humans range from a mild febrile illness to multi-organ system complications [3]. In the agricultural setting, the main manifestations of infections in large animals are reproductive problems, and these result in significant economic losses [6-7]. It is estimated to occur one million cases of leptospirosis per year, resulting in more than 58,000 deaths [8] and 2.90 million disability-adjusted life years (DALYs) [9].

The only commercially available vaccines against leptospirosis are currently bacterins, inactivated whole-cell preparations that induce an immune response that is predominantly against lipopolysaccharide (LPS). As such, protection is limited to the serovars included in the vaccine composition and the immunity induced by bacterins is short term [10]. Multivalent vaccines are required in Brazil, where multiple serovars circulate and epidemiological surveillance is poor, especially with regards to neglected diseases [10]. Besides, bacterins are often associated with adverse reactions and, therefore, these vaccines are not particularly well suited for human use. Thus far, efforts to overcome these limitations have focused on the development of recombinant vaccines based on conserved antigens [11].

Outer membrane proteins are considered promising vaccine targets [12-13]. The proteins LigANI, LigBRep, LipL32, and LemA have been proven to promote partial protection against homologous challenge in hamsters [11, 14-16]. However, such protection does not prevent renal colonization. It may be possible to address this shortfall by fusing a combination of immunogenic antigens in a multi-epitope protein that can provide renal clearance.

Mycobacterium bovis BCG is a live attenuated vaccine that is widely used against tuberculosis and represents a promising alternative for antigen delivery [17]. BCG is safe, stable, and presents strong adjuvant properties. Moreover, it replicates in dendritic cells and macrophages and, therefore, can effectively present antigens to the host immune system and confer long-term immunity [18]. Several heterologous antigens, including those from *Leptospira* spp., have already been successfully expressed in BCG under the control of different promoters [17]. The strength of promoters may affect the levels of antigen expression and strain stability *in vivo* [19]. Recently, our group developed a mycobacterial toolbox (data not published) that contained a stock of compatible sequences constructed using the BioBricks® strategy. This method allows the construction of numerous combinations of parts using a more practical approach than that associated with conventional cloning [20].

In the current study, we report the cloning and expression of two different chimeric genes derived from *Leptospira interrogans* in BCG under the control of five different mycobacterial promoters using previously constructed plasmids that are compatible with the BioBrick method. Moreover, we demonstrate the immunoprotective potential of these rBCG strains as live vaccines against leptospirosis in hamsters.

2. Materials and Methods

2.1. Ethics statement

All animal procedures were performed at the animal facility of the Federal University of Pelotas (UFPel) and approved by the Ethics Committee for Animal Experimentation (CEEA) of UFPel, under protocol number 4646-2015. The CEEA at UFPel is accredited by the Brazilian National Council for Animal Experimentation Control (CONCEA). The animals were maintained in accordance with international guidelines throughout the experiments.

2.2. Strains and growth conditions

Escherichia coli strain DH5α was grown in Luria-Bertani medium at 37 °C with or without kanamycin to 50 µg.ml^{-1}. *Mycobacterium bovis* BCG Pasteur was cultured at 37 ºC in Middlebrook 7H9 medium (Difco, BD, Sao Paulo, SP, Brazil) with 10% of oleic acid, albumin, dextrose complex (OADC - Difco), 0.2% glycerol, and 0.05% Tween 80 or 7H10 agar (Difco, BD, Sao Paulo, SP, Brazil) supplemented with 10% OADC and 0.2% glycerol, with the addition of kanamycin (25 µg.ml^{-1}) when necessary. *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 was grown at 30 ºC in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium supplemented with *Leptospira* enrichment EMJH (Difco, BD, Sao Paulo, SP, Brazil). All experiments with *L. interrogans* were performed using a low-passage strain; i.e., eight passages in hamsters followed by three passages in vitro.

2.3. DNA manipulation reagents

Oligonucleotides were synthesized by Exxtend (Sao Paulo, Brazil). PCR reactions were performed using GoTaq® Colorless Master Mix (Promega, Wisconsin, United States). Restriction enzymes and T4 DNA ligase enzyme were purchased from New England Biolabs (Massachusetts, United States).

2.4. Assembly of BioBrick vectors

BioBrick vectors were previously constructed for antigen expression in BCG (data not published). Briefly, a base plasmid named pUP500 was constructed and contains: origins of replication in *E. coli* and *M. bovis* BCG; a multiple cloning site (MCS) with the prefix and suffix BioBrick-sequences and a resistance gene to kanamycin flanked by restriction sites for *Hind*III. Five different mycobacterial promoters (P_{pAN}, P_{18kDa}, P_{hsp60} and P_{ag85B} with or without signal sequence for secretion – P_{sag85B}) were individually inserted into pUP500 multiple cloning site, generating the following plasmids: pUP500/P_{pAN}, pUP500/P_{18kDa}, pUP500/P_{hsp60}, pUP500/P_{ag85B}, and pUP500/P_{sag85B}. Chimeric genes were amplified by PCR from plasmids previously constructed (data not published) using the primers described in Table 1. Chimeric antigen 1 is a fusion of *lipL32*, *lemA* and *ligANI* (domains 11-13) and chimeric antigen 2 is a fusion of *ligANI* (domains 11-13) and *ligBrep* (Figure 1). BioBrick vectors were digested with *Spe*I and *Pst*I and individually ligated with the chimeric genes digested with *Xba*I and *Pst*I. *E. coli* DH5α competent cells were electroporated with the ten recombinant plasmids and the cloning of chimeric sequences downstream of each promoter into pUP500 was confirmed by sequencing.

2.5. BCG transformation and analysis of expression

M. bovis BCG Pasteur electrocompetent cells were transformed with the recombinant plasmids using the method previously described by Parish & Stoker [21]. BCG transformants were selected in 7H10 medium with kanamycin and grown for 5 days in selective 7H9 medium. Expression of the recombinant proteins was confirmed by Western blot. The cells were then centrifuged at 4000 × g for 10 min, suspended in 1 ml of 100 mM Tris, pH 8.0, and disrupted using a Ribolyser (Hybaid, Kalletal, Germany). The total proteins were separated in SDS-PAGE 10 % and electrotransferred to a nitrocellulose membrane (GE Healthcare, Illinois, United States). Blots were probed with mouse hyperimmune sera produced against each one of the recombinant chimeric proteins (1:500) and peroxidase-conjugated anti-mouse immunoglobulin G (Sigma-Aldrich, Missouri, United States) at a dilution of 1:4000. Detection was carried out using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Illinois, United States).

2.6. Vaccination and challenge of hamsters

Female and male hamsters aged between 4 and 6 weeks were randomly allocated into eleven groups of ten animals per group, equally distributed by gender, as described in Table 2. Animals were subcutaneously immunized with 10^6 CFU of rBCG strains with a 21-day interval between each immunization. A group of four hamsters were immunized in the quadriceps muscle with 10^9 heat-killed whole-leptospires as a positive control. Challenge was performed intraperitoneally fifty-one days after the first immunization with a dose of 10^3 leptospires, equivalent to five times the 50% lethal dose (LD_{50}) of the *L. interrogans* sv. Copenhageni strain Fiocruz L1-130. Before each immunization and challenge, blood samples were collected from the retro-orbital venous plexus with administration of anesthetic eye drops, and the sera were stored at -20°C.

Animals were humanly euthanized by deep anesthesia using pentobarbital when the end-point criteria were reached. These criteria consisted of loss of appetite, gait difficulty, prostration, dyspnea, ruffled fur, and weight loss of 10% of the animal's maximum weight [22]. Survivors were euthanized 30 days post challenge.

2.7. Humoral immune response determination

The antibody response was evaluated by indirect ELISA using purified chimeric proteins. Each protein was used separately in a concentration of 500 ng per well, diluted in carbonate-bicarbonate buffer, pH 9.6. The ELISA plates were blocked with 5% fat-free dry milk, and hamster's sera was added at a 1:50 dilution in PBST (PBS with 0.05% [v/v] Tween 20) for 1 h at 37°C. Peroxidase-conjugated anti-golden Syrian hamster IgG antibody (Rockland Immunochemicals, Pennsylvania, United States) was added at a 1:6,000 dilution and incubation proceeded at 37°C for 1 h. Washing with PSBT was performed between all steps. The reaction was developed by adding o-phenylenediamine dihydrochloride (Sigma-Aldrich, Missouri, United States) and hydrogen peroxide. The reaction was stopped with 25 µl of 4 N

H_2SO_4, and the absorbance was read at 492 nm. Mean values were calculated from serum samples assayed in triplicate.

2.8. Analysis of leptospiral presence in kidneys

The presence or absence of leptospires in the kidney samples of the surviving animals was evaluated by culture and real-time quantitative PCR (qPCR). Leptospiral genomic DNA in the kidney samples was quantified by qPCR using a LightCycler 96 system (Roche, Basel, Switzerland). Samples were prepared by dicing 100-200 mg of kidney tissue and suspending it in PBS, followed by tissue homogenization for two cycles of 20 s using a Ribolyser (Hybaid, Kalletal, Germany). Genomic DNA was extracted from approximately 40 mg of tissue using the SV Genomic DNA Purification kit (Promega, Brazil). Reactions were performed in triplicate usingGoTaq Probe qPCR Master Mix (Promega) with the primers and probes described in Table 1. The cycling parameters were denaturation at 95°C for 10 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/elongation for 60 s at 60°C. The number of leptospires was determined in comparison to the number of hamster cells in the sample, quantified by copies of the *lipl32* and *β-actin* genes respectively. For culture, kidney samples were collected and inoculated in EMJH medium. During an eight-week incubation period, dark-field microscopy was performed to identify positive cultures.

2.9. Statistical analysis

Protection against mortality and the survival rates were determined using the Fisher's exact test and log-rank test respectively. Significant differences between the serological assays were determined by analysis of variance (ANOVA). $P \leq 0.05$ was considered to be statistically significant. Statistical analysis was carried out using the GraphPad Prism 7 and Statistix 8 software packages.

3. Results

3.1. Construction of rBCG expressing chimeric proteins of *Leptospira interrogans*

The expression of both chimeric proteins in BCG under the control of five different mycobacterial promoters was evaluated by Western blot (Figure 2). The polyclonal antibodies produced against each chimeric protein (anti-Q1 or anti-Q2) were able to specifically recognize their respective antigenic portions; i.e., LipL32, LigAni, LigBrep, and LemA (data not shown). Serum anti-Q1 and anti-Q2 were able to recognize chimeric proteins 1 and 2, with approximately 75 and 84 kDa respectively in rBCG cell lysates. No expression was detected in wild-type BCG extract used as negative control.

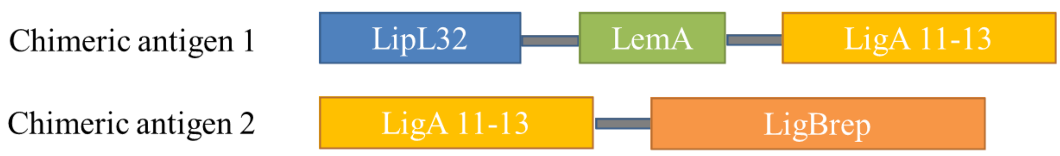


Fig. 1 Schematic illustration of chimeric proteins evaluated in this study, constructed from the combination of immunogenic antigens from *Leptospira interrogans*. Chimeric antigen 1 is a fusion of *lipL32*, *lemA* and *ligANI* (domains 11-13) and chimeric antigen 2 is a fusion of *ligANI* (domains 11-13) and *ligBrep*.

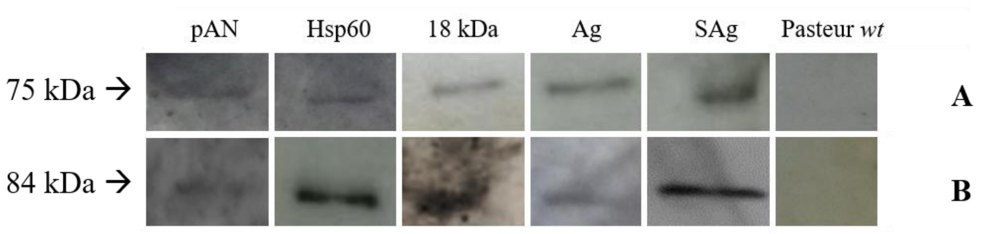


Fig. 2 Western blot demonstrating the expression of chimeric antigens in *M. bovis* BCG. Characterization of chimeric antigen 1 (A) and 2 (B) expressed in BCG under control of different mycobacterial promoters, as indicated. Pasteur *wt*, wild-type used as negative control.

3.2. Antibody response elicited by rBCG vaccines

The antibody response induced by rBCG vaccines was evaluated by ELISA, and the results are summarized in Figure 3. The humoral response induced by rBCG vaccines based on chimera 1 was significantly higher on day 21 than pre-immune day 0, and decreased on day 51. Significant seroconversion ($P \leq 0.05$) was observed in hamsters vaccinated with rBCG strains in which the expression of chimera 1 was controlled by promoters pAN, 18 kDa, and Ag85B plus signal sequence. Expression of chimera 1 in BCG under the control of Hsp60 and Ag85B promoters did not induce significant levels of IgG (P >0.05).

All animals immunized with rBCG vaccines expressing chimera 2 produced significantly higher levels of IgG than those in the control group. However, no statistical difference was observed among vaccine groups. This response was maintained even after the second dose (day 51), and its magnitude was the same on day 21 (P >0.05).

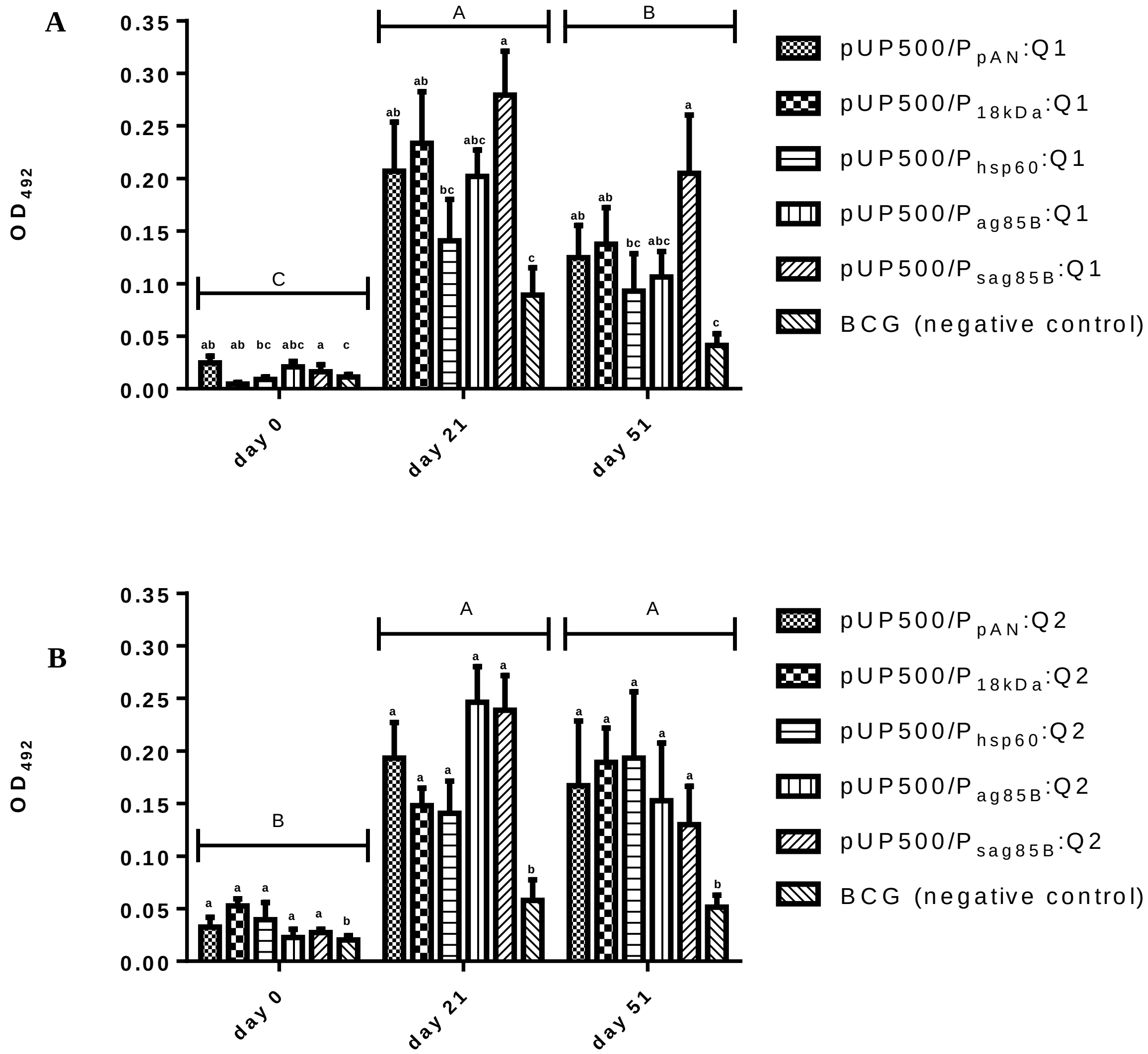
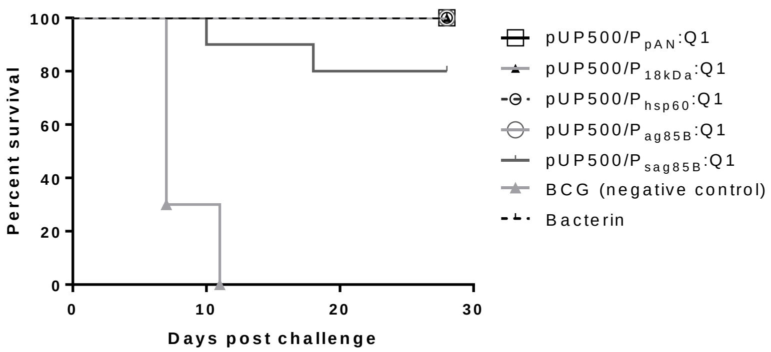


Fig. 3 Specific IgG response in hamsters immunized with rBCG vaccines. Recombinant chimeric proteins expressed by *E. coli* were used as the antigen in ELISA. (A) IgG levels induced by rBCG vaccines based on chimeric antigen 1 (Q1). (B) IgG levels induced by rBCG vaccines based on chimeric antigen 2 (Q2). The results represent mean absorbance ± standard deviation calculated from serum samples assayed in triplicate. OD_{492}, optical density at 492 nm. The significance was determined by the analysis of variance (Tukey multiple comparison). Groups without common lower case letters differ (*P*< 0.05); days without common upper case letters differ (*P*< 0.05).

3.3. Protective efficacy of rBCG strains in hamster model

The immunoprotective potential of rBCG strains against leptospirosis was evaluated in terms of survival, recorded 30 days after challenge. The results of the mortality test (Fisher test) and the survival rates are shown in Table 2 and Figure 4 respectively. All rBCG strains expressing the chimeric antigen 1 afforded 100% protection against challenge except for the strain in which chimera 1 was secreted, which conferred 80% protection (P <0.05). Among the rBCG strains expressing the chimeric antigen 2, only the one using the pAN promoter afforded significant protection against challenge (60% of survival). All animals vaccinated with bacterin survived after challenge. End-point criteria were observed in all animals from the negative control group, which received the wild-type BCG Pasteur.

A



B

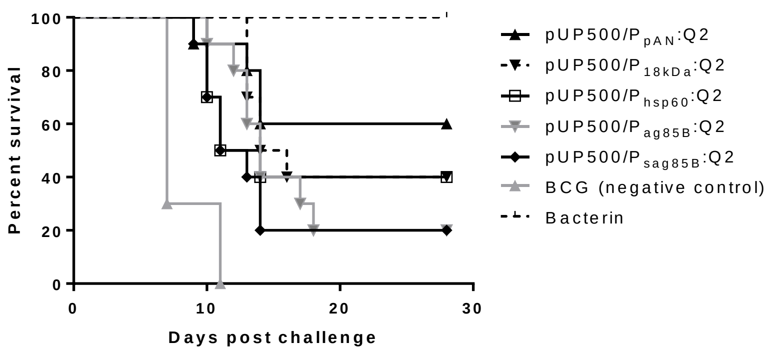


Fig. 4 Survival of hamsters immunized with rBCG after lethal challenge. Survival among hamsters vaccinated with rBCG strains or bacterin, after the administration of a potentially lethal inoculum of *L. interrogans* serovar Copenhageni strain Fiocruz L1–130. (A) rBCG vaccines based on chimeric antigen 1 (Q1). (B) rBCG vaccines based on chimeric antigen 2 (Q2). Survival curves were compared using log-rank analysis.

3.4. Sterile immunity induced by rBCG vaccines

Culture isolation and qPCR were used to evaluate whether vaccines were able to prevent renal colonization. All kidney cultures collected from the surviving animals immunized with rBCG strains expressing chimeric antigen 1 were negative. In qPCR, these vaccines did not prevent renal colonization in one animal from the group that received this antigen expressed under the control of Hsp60 promoter (1/10) and in all animals that received the construct using Ag85B promoter plus signal sequence (8/8). All survivors from the groups immunized with vaccines based on chimeric protein 2 were qPCR-positive. Animals from the negative control group were positive in culture (4/10) and in qPCR (9/10) (data not shown). Two of the three animals vaccinated with bacterin were also positive in qPCR (Table 2).

4. Discussion

Several proteins have exhibited potential as vaccine candidates against leptospirosis. The most evaluated antigens so far are the Lig proteins and the lipoprotein LipL32 [11]. Despite reports of protection, in most experiments, the animals remained chronic renal carriers. The hypothetical lipoprotein LemA also elicited partial protection against challenge, in prime-boost and DNA vaccination strategies, although the induction of sterilizing immunity has also not been reported [15]. Humoral immune response is known to be responsible for protection. However, in cattle, cellular immunity also plays a fundamental role in promoting protective immunity to *Leptospira* infection [23-25]. *M. bovis* BCG is a potent cellular immunity enhancer [17]. Thus, rBCG may represent an attractive vaccine vehicle for the expression of leptospiral antigens. We have recently developed a kit to work with mycobacteria that was standardized according to BioBrick strategy [20] (data not published).

In this study, the expression of chimeric antigens containing sequences from *L. interrogans* in rBCG was achieved under the control of five different promoters using the previously constructed BioBrick vectors. The first chimeric antigen includes amino acid sequences of LipL32, LemA, and LigANI, and was able to confer significant protection against challenge (80-100%) when expressed in rBCG under the control of any of the five promoters tested. Eighty percent of survival was observed when the Ag85B promoter plus a signal sequence was used. The reduction in this rate in comparison to the other groups might be explained by the secretion of the antigen to the extracellular medium [26]. The other chimeric antigen evaluated was a fusion of LigANI and LigBrep and was only able to protect hamsters when expressed under the control of pAN promoter. This could be attributed to the fact that this construction has a superior stability due to the strength of promoter, as demonstrated in other studies [27-28]. Both chimeric proteins were protective when evaluated as recombinant subunit vaccines; however, they did not confer sterile immunity (data not published). Using BCG as a vector, the chimeric antigen 1 presents a more effective combination of sequences to achieve protection than the second chimeric antigen. Recently, a subunit vaccine based on LigBrep was shown to be protective [14]. Surprisingly, the rBCG vaccines that afforded protection in the current study were predominantly based on chimeric antigen 1, which does not present LigBrep in its sequence. This might be due to the size of the antigens; chimeric antigen 2 presents a higher molecular weight, which may have been costly for its expression in BCG, reducing the bacterial fitness and resulting in the loss of the heterologous antigen. The instability of the rBCG strain expressing OspA antigen was possibly a determinant of the lack of protection against *Borrelia burgdorferi* [29].

There is no immune correlate established for leptospirosis that can be associated with protection [30]. Here, we evaluated the humoral immune response by ELISA and observed significant levels of IgG induced by rBCG vaccines with only a single dose. Vaccines in which the promoters pAN, 18 kDa and Ag85B plus a signal sequence, commanded the expression of chimera 1 were able to induce a significant IgG response. Chimera 2 was able to induce antibodies when expressed by any of the promoters evaluated; however, protection was only achieved with the pAN promoter. Despite antibody production, several recombinant subunit vaccines did not confer protection against leptospirosis [11]. In light of its adjuvant potential, the use of BCG may represent a potential strategy by which it is possible to strengthen the immune response of recombinant antigens. Analysis of the cellular response will be of importance, especially when using BCG as a vaccine vector; however, this is hampered by the lack of knowledge and tools that are available to characterize hamster immunity.

It is well known that culture isolation is not the most reliable method of detecting the presence of leptospires in kidneys [31]. Thus, we also performed a quantitative real-time PCR using a specific probe for *lipL32* to evaluate the sterile immunity induced by rBCG vaccine preparations. We believe that the use of specific probes in qPCR is more reliable, sensitive, and specific than other methods such as imprint and qPCR based on SYBR green. The lack of a definitive and standardized tool to determine leptospiral colonization makes the association of different methods as the most rational approach to confirm bacterial burden in kidneys [32]. Until now, sterilizing immunity has been reported only for a LigBrep-based subunit vaccine [14]. Here, we demonstrated that four of the five rBCG vaccines based on chimeric protein 1 protected hamsters against challenge and renal colonization.

To the best of our knowledge, this study is the first of its kind to demonstrate the potential of chimeric antigens in live vaccines vectorized by BCG against leptospirosis. We also validated the application of BioBrick vectors in the expression of heterologous antigens in BCG, which can be expanded to other pathogens of interest.

5. Conclusions

Our results highlight the potential of BCG as a vector to deliver antigens from *Leptospira* and the construction of chimeric genes as a promising alternative to the development of an effective vaccine against leptospirosis. Further studies are required to investigate whether these vaccines are able to protect animals against different serovars of *Leptospira* that are introduced via a natural route of infection.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Authors' Contributions

Conceived and designed the experiments: TLO, CEPC, CR, OAD. Performed the experiments: TLO, CR, JD, ACPSN, MA. Analyzed the data: TLO, CEPC, CR. Wrote the paper: TLO, OAD. Final approval of the version to be submitted: TLO, CEPC, CR, JD, ACPSN, MA, OAD.

Acknowledgements

We are grateful to Michele dos Santos for the technical assistance provided during this study. This work was funded by grants from CNPq, CAPES and FAPERGS, Brazilian Research Funding Agencies.

References

[1] Adler B, de la Pena Moctezuma A. *Leptospira* and leptospirosis. Vet Microbiol 2010;140:287-96.

[2] Ko AI, Goarant C, Picardeau M. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol 2009;7:736-47.

[3] Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol 2015;387:65-97.

[4] Maciel EA, de Carvalho AL, Nascimento SF, de Matos RB, Gouveia EL, Reis MG, et al. Household transmission of *leptospira* infection in urban slum communities. PLoS Negl Trop Dis 2008;2:e154.

[5] Reis RB, Ribeiro GS, Felzemburgh RD, Santana FS, Mohr S, Melendez AX, et al. Impact of environment and social gradient on *Leptospira* infection in urban slums. PLoS Negl Trop Dis 2008;2:e228.

[6] Lilenbaum W, Martins G. Leptospirosis in cattle: a challenging scenario for the understanding of the epidemiology. Transbound Emerg Dis 2014;61 Suppl 1:63-8.

[7] Ellis WA. Animal leptospirosis. Curr Top Microbiol Immunol 2015;387:99-137.

[8] Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Negl Trop Dis 2015;9:e0003898.

[9] Torgerson PR, Hagan JE, Costa F, Calcagno J, Kane M, Martinez-Silveira MS, et al. Global Burden of Leptospirosis: Estimated in Terms of Disability Adjusted Life Years. PLoS Negl Trop Dis 2015;9:e0004122.

[10] Adler B. Vaccines against leptospirosis. Curr Top Microbiol Immunol 2015;387:251-72.

[11] Dellagostin OA, Grassmann AA, Hartwig DD, Felix SR, da Silva EF, McBride AJ.

Recombinant vaccines against leptospirosis. Hum Vaccin 2011;7:1215-24.

[12] Grassmann AA, Kremer FS, Dos Santos JC, Souza JD, Pinto LDS, McBride AJA. Discovery of Novel Leptospirosis Vaccine Candidates Using Reverse and Structural Vaccinology. Front Immunol 2017;8:463.

[13] Haake DA, Zuckert WR. The leptospiral outer membrane. Curr Top Microbiol Immunol 2015;387:187-221.

[14] Conrad NL, Cruz McBride FW, Souza JD, Silveira MM, Felix S, Mendonca KS, et al. LigB subunit vaccine confers sterile immunity against challenge in the hamster model of leptospirosis. PLoS Negl Trop Dis 2017;11:e0005441.

[15] Hartwig DD, Forster KM, Oliveira TL, Amaral M, McBride AJ, Dellagostin OA. A prime-boost strategy using the novel vaccine candidate, LemA, protects hamsters against leptospirosis. Clin Vaccine Immunol 2013;20:747-52.

[16] Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, et al. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine 2007;25:6277-86.

[17] Bastos RG, Borsuk S, Seixas FK, Dellagostin OA. Recombinant *Mycobacterium bovis* BCG. Vaccine 2009;27:6495-503.

[18] Nasser Eddine A, Kaufmann SH. Improved protection by recombinant BCG. Microbes Infect 2005;7:939-46.

[19] Oliveira TL, Rizzi C, Dellagostin OA. Recombinant BCG vaccines: molecular features and their influence in the expression of foreign genes. Appl Microbiol Biotechnol 2017.

[20] Shetty RP, Endy D, Knight TF, Jr. Engineering BioBrick vectors from BioBrick parts. J Biol Eng 2008;2:5.

[21] Parish T, Stoker NG. Electroporation of mycobacteria. Methods Mol Biol 1995;47:237-

52.

[22] Coutinho ML, Choy HA, Kelley MM, Matsunaga J, Babbitt JT, Lewis MS, et al. A LigA Three-Domain Region Protects Hamsters from Lethal Infection by *Leptospira interrogans*. PLoS Negl Trop Dis 2011;5:e1422.

[23] Babiuk LA, Babiuk SL, Loehr BI, van Drunnen Littel-van den H. Nucleic acid vaccines: research tool or commercial reality. Vet Immunol Immunopathol 2000;76:1-23.

[24] Bolin CA, Alt DP. Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to *Leptospira borgpetersenii* serovar hardjo. Am J Vet Res 2001;62:995-1000.

[25] Naiman BM, Alt D, Bolin CA, Zuerner R, Baldwin CL. Protective killed *Leptospira borgpetersenii* vaccine induces potent Th1 immunity comprising responses by CD4 and gammadelta T lymphocytes. Infect Immun 2001;69:7550-8.

[26] Kaufmann SH, Hess J. Impact of intracellular location of and antigen display by intracellular bacteria: implications for vaccine development. Immunol Lett 1999;65:81-4.

[27] Medeiros MA, Dellagostin OA, Armoa GR, Degrave WM, De Mendonca-Lima L, Lopes MQ, et al. Comparative evaluation of *Mycobacterium vaccae* as a surrogate cloning host for use in the study of mycobacterial genetics. Microbiology 2002;148:1999-2009.

[28] Seixas FK, da Silva EF, Hartwig DD, Cerqueira GM, Amaral M, Fagundes MQ, et al. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. Vaccine 2007;26:88-95.

[29] Edelman R, Palmer K, Russ KG, Secrest HP, Becker JA, Bodison SA, et al. Safety and immunogenicity of recombinant Bacille Calmette-Guerin (rBCG) expressing *Borrelia burgdorferi* outer surface protein A (OspA) lipoprotein in adult volunteers: a candidate Lyme disease vaccine. Vaccine 1999;17:904-14.

[30] Zuerner RL. Host response to leptospira infection. Curr Top Microbiol Immunol

2015;387:223-50.

[31] Cameron CE. Leptospiral structure, physiology, and metabolism. Curr Top Microbiol Immunol 2015;387:21-41.

[32] Chagas-Junior AD, da Silva CL, Soares LM, Santos CS, Silva CD, Athanazio DA, et al. Detection and quantification of *Leptospira interrogans* in hamster and rat kidney samples: immunofluorescent imprints versus real-time PCR. PLoS One 2012;7:e32712.

Table 1. Nucleotide sequences of primers and probes used in this work.

Primer/probe	**Sequence (5’-3’)**	**Label**	**Final concentration (nM)**
Chi1 forward	CCGGAATTCGCGGCCGCTTCTAGAGGTGGTCTGCCAAGCCTAA	-	500
Chi1 reverse	TGCACTGCAGCGGCCGCTACTAGTTTATTATGGCTCCGTTTTAATAGA	-	500
Chi2 forward	CCGGAATTCGCGGCCGCTTCTAGAAGAATAGCTTCAATC	-	500
Chi2 reverse	TGCACTGCAGCGGCCGCTACTAGTTTATGGAGTGAGTGTATT	-	500
lipl32 forward	TTGGATCCGTGTAGAAAGAATGTC	-	300
lipl32 reverse	TCGTCCAATTTTTGAACTGGTTT	-	300
lipl32 probe	CCAAATCGCCAAAGCTGCGAAAGC	FAM/ZEN/IowaBlack	250
β-actin forward	TTCAACACCCCWGCCATGTA	-	300
β-actin reverse	TCWCCGGAGTCCATCACRAT	-	600
β-actin probe	CCATCCAGGCYGTGCTGTCCCTG	VIC/ZEN/IowaBlack	250

Table 2. Protection conferred by different rBCG-vectorized vaccines against lethal challenge in the hamster model of leptospirosis.

Immunogen[a]	% Protection (survivors/total)	*P* value[b]	Renal culture (positive/survivors)	qPCR (positive/survivors)
rBCG (pUP500/P_{pAN}:chimericprotein1)	100 (10/10)	< 0.0001	0/10	0/10
rBCG (pUP500/P_{18kDa}:chimericprotein1)	100 (10/10)	< 0.0001	0/10	0/10
rBCG (pUP500/P_{hsp60}:chimericprotein1)	100 (10/10)	< 0.0001	0/10	1/10
rBCG (pUP500/P_{ag85B}:chimericprotein1)	100 (10/10)	< 0.0001	0/10	0/10
rBCG (pUP500/P_{sag85B}:chimericprotein1)	80 (8/10)	0.0007	0/8	8/8
rBCG (pUP500/P_{pAN}:chimericprotein2)	60 (6/10)	0.0108	3/6	6/6
rBCG (pUP500/P_{18kDa}:chimericprotein2)	40 (4/10)	0.0867	3/4	4/4
rBCG (pUP500/P_{hsp60}:chimericprotein2)	40 (4/10)	0.0867	1/4	4/4
rBCG (pUP500/P_{ag85B}:chimericprotein2)	20 (2/10)	0.4737	0/2	2/2
rBCG (pUP500/P_{sag85B}:chimericprotein2)	20 (2/10)	0.4737	0/2	2/2
Bacterin (positive control)	100 (3/3)	0.0035	0/3	2/3
BCG Pasteur (negative control)	0 (0/10)	-	-	-

[a] Chimeric protein 1 is a fusion of *lipL32*, *lemA* and *ligANI* (domains 11-13); chimeric protein 2 is a fusion of *ligANI* (domains 11-13) and *ligBrep*.

[b] Two-tailed *P* value determined by Fisher exact test in comparison to the negative control group.

4 CONCLUSÃO GERAL

- A aplicação da tecnologia BioBricks para a clonagem de sequências de *Mycobacterium bovis* BCG é uma metodologia promissora e inovadora para a manipulação genética deste microrganismo.
- Os vetores construídos e caracterizados neste estudo são funcionais e tornam-se disponíveis para a expressão de antígenos de quaisquer microrganismos em BCG, utilizando a tecnologia BioBricks.
- A utilização de *egfp* como gene repórter é uma ferramenta útil para caracterização dos vetores construídos e permite além da validação da técnica, a avaliação da atividade de diferentes promotores micobacterianos em BCG cultivado *in vitro* e dentro de macrófagos.
- A expressão de quimeras recombinantes de *Leptospira interrogans* em BCG utilizando os vetores construídos demonstra a aplicação do sistema desenvolvido nesse trabalho para expressão de antígenos de diferentes patógenos.
- As vacinas vetorizadas por BCG construídas são promissoras para o controle e prevenção da leptospirose.

5 REFERÊNCIAS

BASTOS, R. G., BORSUK, S., SEIXAS, F. K. & DELLAGOSTIN, O. A. (2009). Recombinant *Mycobacterium bovis* BCG. *Vaccine*. 27**.** p. 6495-6503.

CASINI, A., STORCH, M., BALDWIN, G. S. & ELLIS, T. (2015). Bricks and blueprints: methods and standards for DNA assembly. *Nat Rev Mol Cell Biol*. 16**.** p. 568-576.

CONSTANTE, M., GRUNBERG, R. & ISALAN, M. (2011). A biobrick library for cloning custom eukaryotic plasmids. *PLoS One*. 6**.** p. e23685.

KAUFMANN, S. H. & HESS, J. (1999). Impact of intracellular location of and antigen display by intracellular bacteria: implications for vaccine development. *Immunol Lett*. 65**.** p. 81-84.

LEE, C. F., CHANG, S. Y., HSIEH, D. S. & YU, D. S. (2004). Treatment of bladder carcinomas using recombinant BCG DNA vaccines and electroporative gene immunotherapy. *Cancer Gene Ther*. 11**.** p. 194-207.

LEVSKAYA, A., CHEVALIER, A. A., TABOR, J. J., SIMPSON, Z. B., LAVERY, L. A., LEVY, M., DAVIDSON, E. A., SCOURAS, A., ELLINGTON, A. D., MARCOTTE, E. M. & VOIGT, C. A. (2005). Synthetic biology: engineering *Escherichia coli* to see light. *Nature*. 438**.** p. 441-442.

MATSUO, K., YAMAGUCHI, R., YAMAZAKI, A., TASAKA, H., TERASAKA, K., TOTSUKA, M., KOBAYASHI, K., YUKITAKE, H. & YAMADA, T. (1990). Establishment of a Foreign Antigen Secretion System in *Mycobacteria*. *Infection and Immunity*. 58**.** p. 4049-4054.

MATSUO, K. & YASUTOMI, Y. (2011). *Mycobacterium bovis* Bacille Calmette-Guerin as a Vaccine Vector for Global Infectious Disease Control. *Tuberc Res Treat*. 2011**.** p. 574591.

MULLER, K. M. & ARNDT, K. M. (2012). Standardization in synthetic biology. *Methods Mol Biol*. 813**.** p. 23-43.

OHARA, N. & YAMADA, T. (2001). Recombinant BCG vaccines. *Vaccine*. 19**.** p. 4089-4098.

OLIVEIRA, T. L., RIZZI, C. & DELLAGOSTIN, O. A. (2017). Recombinant BCG vaccines: molecular features and their influence in the expression of foreign genes. *Appl Microbiol Biotechnol*. p.

POPP, P. F., DOTZLER, M., RADECK, J., BARTELS, J. & MASCHER, T. (2017). The Bacillus BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with *Bacillus subtilis*. *Sci Rep*. 7**.** p. 15058.

QUAN, Y., JEONG, C. W., KWAK, C., KIM, H. H., KIM, H. S., KU, J. H. (2017). Dose, duration and strain of bacillus Calmette-Guerin in the treatment of nonmuscle invasive bladder cancer: Meta-analysis of randomized clinical trials. *Medicine (Baltimore)*. 96 (42). p. 8300.

RADECK, J., KRAFT, K., BARTELS, J., CIKOVIC, T., DURR, F., EMENEGGER, J., KELTERBORN, S., SAUER, C., FRITZ, G., GEBHARD, S. & MASCHER, T. (2013). The *Bacillus* BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*. *J Biol Eng*. 7**.** p. 29.

SHETTY, R. P., ENDY, D. & KNIGHT, T. F., JR. (2008). Engineering BioBrick vectors from BioBrick parts. *J Biol Eng*. 2**.** p. 5.

SINGH, V. K., SRIVASTAVA, R. & SRIVASTAVA, B. S. (2016). Manipulation of BCG vaccine: a double-edged sword. *Eur J Clin Microbiol Infect Dis*. 35**.** p. 535-543.

SMOLKE, C. D. (2009). Building outside of the box: iGEM and the BioBricks Foundation. *Nat Biotechnol*. 27**.** p. 1099-1102.

SUREKA, S., CHAKRAVORTY, A., HOLMES, E. C., SPASSIBOJKO, O., BHATT, N., WU, D. & TURGEON, B. G. (2014). Standardization of functional reporter and antibiotic resistance cassettes to facilitate the genetic engineering of filamentous fungi. *ACS Synth Biol*. 3**.** p. 960-962.

6 ANEXOS

Anexo A - Pedido de Patente

(Depositada junto ao Instituto Nacional da Propriedade Industrial)

VETORES BIOBRICKS PARA CONSTRUÇÃO DE MICOBACTÉRIAS RECOMBINANTES.

Inventores: Thaís Larré Oliveira
Carlos Eduardo Pouey da Cunha
Caroline Rizzi
Odir Antônio Dellagostin

Resumo da invenção:

"Vetores BioBricks para construção de micobactérias recombinantes" refere-se à construção e à clonagem de sequências de DNA, frequentemente utilizadas para obtenção de cepas de BCG recombinante e vetores plasmideais para utilização em *Mycobacterium* spp., através do método BioBricks. O método BioBricks tem por objetivo padronizar a clonagem e a montagem de moléculas de DNA. Esta padronização ocorre pela utilização de sequências prefixo e sufixo flanqueando os fragmentos alvo, as quais contêm sítios para enzimas de restrição; ao clivarem seus sítios alvo, tais enzimas geram extremidades compatíveis entre si. Sendo assim, torna-se possível a ligação de uma parte à outra de maneira adjacente, formando novas moléculas que conservam em suas extremidades todos os sítios originais. A presente invenção refere-se aos métodos para obtenção de fragmentos de DNA padronizados e compatíveis para o desenvolvimento de cepas de micobactérias recombinantes, por exemplo BCG recombinante, expressando antígenos de interesse, bem como aos produtos obtidos pelos referidos métodos.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

18/10/2017 870170079281 13:30

29409161708436226

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 022407 4

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social:	UNIVERSIDADE FEDERAL DE PELOTAS
Tipo de Pessoa:	Pessoa Jurídica
CPF/CNPJ:	92242080000100
Nacionalidade:	Brasileira
Qualificação Jurídica:	Instituição de Ensino e Pesquisa
Endereço:	Rua Gomes Carneiro, 01 - Ed. Delfim Mendes Silveira - Campus Porto/Reitoria - 4° Andar - PRPPG
Cidade:	Pelotas
Estado:	RS
CEP:	96010-610
País:	Brasil
Telefone:	(53) 3284 4086
Fax:	
Email:	cit@ufpel.edu.br

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): VETORES BIOBRICKS PARA CONSTRUÇÃO DE MICOBACTÉRIAS RECOMBINANTES

Resumo: A patente refere-se à construção e à clonagem de sequências de DNA, frequentemente utilizadas para obtenção de cepas de BCG recombinante e vetores plasmideais para utilização em Mycobacterium spp., através do método BioBricks. O método BioBricks tem por objetivo padronizar a clonagem e a montagem de moléculas de DNA. Esta padronização ocorre pela utilização de sequências prefixo e sufixo flanqueando os fragmentos alvo, as quais contêm sítios para enzimas de restrição; ao clivarem seus sítios alvo, tais enzimas geram extremidades compatíveis entre si. Sendo assim, torna-se possível a ligação de uma parte à outra de maneira adjacente, formando novas moléculas que conservam em suas extremidades todos os sítios originais. A presente invenção refere-se aos métodos para obtenção de fragmentos de DNA padronizados e compatíveis para o desenvolvimento de cepas de micobactérias recombinantes, por exemplo BCG recombinante, expressando antígenos de interesse, bem como aos produtos obtidos pelos referidos métodos.

Figura a publicar: 1

Anexo B - Pedido de Patente

(Enviada para aprovação da Coordenação de Inovação Tecnológica da UFPel)

PROTEÍNAS QUIMÉRICAS COMO ANTÍGENOS VACINAIS CONTRA LEPTOSPIROSE

Inventores: Carlos Eduardo Pouey da Cunha
Thaís Larré Oliveira
Caroline Rizzi
Daiane Drawanz Hartwig
Odir Antônio Dellagostin

Resumo da invenção:

"Proteínas quiméricas como antígenos vacinais contra leptospirose"refere-se à construção de antígenos quiméricos contendo diferentes combinações de sequências de proteínas imunogênicas de *Leptospira* spp. A patente também se refere à avaliação destas quimeras como vacinas de subunidade recombinante e vacinas vetorizadas por *Mycobacterium bovis* BCG contra leptospirose em hamsters.