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Tese

Construção de marcador auxotrófico em *Mycobacterium bovis* BCG, de uma cepa de BCG *knockout* para o antígeno DPPD e estudo proteômico da tuberculina

Sibele Borsuk

Pelotas, 2008

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia Agrícola da Universidade Federal de Pelotas, como requisito parcial à obtenção do titulo de Doutor em Ciências (Área de Conhecimento: Biologia Molecular).

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Prof. Dr. Fabricio Rochedo Conceição, Fundação Universidade do Rio Grande

Prof. Dr. Itabajara da Silva Vaz Junior, Universidade Federal do Rio Grande do Sul

Prof.Dr. Pedro Almeida da Silva, Fundação Universidade do Rio Grande

Prof. Dr. Odir Antônio Dellagostin, Universidade Federal de Pelotas

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RESUMO

BORSUK, Sibele. **Construção de marcador auxotrófico em *Mycobacterium bovis* BCG, de uma cepa de BCG *knockout* para DPPD e estudo proteômico da tuberculina**. 2008. 114 f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia Agrícola. Universidade Federal de Pelotas, Pelotas.

Mycobacterium bovis BCG tem o potencial para ser um vetor efetivo para vacinas recombinantes multivalentes. No entanto, existem dois problemas quanto a sua utilização como vetor vacinal. O primeiro é a presença de genes que conferem resistência a antibióticos nos vetores utilizados para transformação genética. O segundo é a limitação de uso de BCG em animais, principalmente por comprometer o teste de tuberculina, utilizado como diagnóstico de tuberculose, o qual se baseia em reação de hipersensibilidade ao PPD (Derivado Protéico Purificado). Neste trabalho desenvolvemos e avaliamos a complementação auxotrófica como novo marcador de seleção, fizemos a caracterização das proteínas componentes de amostras de PPD aviário e bovino e desenvolvemos um mutante de BCG por recombinação homóloga. Para o uso de complementação auxotrófica como marcador de seleção, uma cepa de BCG auxotrófica para o aminoácido leucina foi construída por *knockout* do gene *leuD* por recombinação homóloga. A expressão do gene *leuD* em um plasmídio atuou como marcador de seleção nas cepas auxotróficas de *M. bovis* BCG Δ*leu*D e *M. smegmatis* mc^2144. A seleção por complementação de BCG auxotrófica se mostrou equivalente à seleção por resistência a antibiótico, com a vantagem adicional de proporcionar maior estabilidade do vetor plasmidial, já que a pressão seletiva é mantida mesmo durante multiplicação da bactéria *in vivo*. A identificação das proteínas que compõem o PPD foi feita por espectrometria de massa utilizando-se LC-MS/MS (cromatografia líquida associada à espectrometria de massa em tandem). Foram identificadas 147 proteínas entre 5 amostras de PPD (2 PPD bovino e 3 PPD aviário). O PPD bovino teve um número maior de proteínas comparado ao PPD aviário. Foi identificado um grupo de 28 proteínas presentes em PPD bovino, mas ausentes em PPD aviário. Além disso, 5 proteínas encontradas no PPD estão ausentes em *M. bovis* BCG. Estes são de

especial interesse, pois poderão vir a contribuir para o desenvolvimento de um teste de diagnóstico mais específico, e possivelmente capaz de diferenciar indivíduo vacinado com BCG e infectado com o bacilo da tuberculose. Um mutante de *M. bovis* BCG Pasteur foi construído. O gene Mb0092 (*dppd*) foi alvo de inativação gênica por recombinação homóloga. Seqüências que flanqueiam o gene alvo foram clonadas em um vetor suicida. Duplo *crossover* foi selecionado utilizando *sac*B. O genótipo mutante foi determinado por PCR e por Southern blot. Esta cepa poderá ser utilizada como vacina em animais, quando o diagnóstico for feito com DPPD recombinante. Os resultados obtidos apresentam alternativas para os problemas envolvidos quanto à utilização de *M. bovis* BCG como vacina recombinante. O sistema de seleção por complementação auxotrófica foi estável, e pode ser empregado na expressão de antígenos heterólogos em BCG. A identificação dos principais componentes protéicos do PPD e o desenvolvimento da cepa mutante de BCG possibilitam o desenvolvimento de testes diagnósticos diferencias, permitindo a utilização de BCG como vacina também em animas.

Palavras chave: BCG recombinante, complementação auxotrófica, caracterização PPD.

ABSTRACT

BORSUK, Sibele. **Construction of auxotrophic marker in *Mycobacterium bovis* BCG, knockout strain for the DPPD and proteomic study of tuberculin.** 2008. 114 f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia Agrícola. Universidade Federal de Pelotas, Pelotas.

Mycobacterium bovis BCG has the potential to be an effective live vector for multivalent vaccines. However, there are two problems regarding the utilization of recombinant BCG as vaccine. The first one is that most mycobacterial cloning vectors rely on antibiotic resistance gene as selectable marker, which is used for genetic transformation. The second one is the limited use of BCG in animals because it interferes in the tuberculosis diagnosis by tuberculin skin test, which elicits delayed type hypersensitivity to the purified protein derivative (PPD). In this work we developed and evaluated the use of auxotrophic complementation as a new selectable marker, characterized the proteins that are present in the bovine and avium PPD and developed a knockout BCG strain by homologous recombination. To test the auxotrophic complementation as selectable marker, an auxotrophic BCG strain for the amino acid leucine was constructed by knocking out the *leuD* gene by homologous recombination. Expression of *leuD* on a plasmid acted as a selectable marker in the auxotrophic *M. bovis* BCG Δ*leuD* and *M. smegmatis* mc^2144. The auxotrophic complementation selection was similar to selection by antibiotic resistance, but with the advantage of promoting stability of the plasmid. The new system was highly stable even during *in vivo* BCG growth. The identification of proteins from PPD was archived by LC-MS/MS (Liquid Chromatography/Mass Spectrometry/Mass Spectrometry). A total of 147 proteins among five PPD samples (2 bovine PPD and 3 avium PPD) were identified. The bovine PPD had a considerable higher number of proteins comparing to the avium PPD. We identifying a group of 28 proteins present only in bovine PPD and a group of five proteins deleted in *M. bovis* BCG vaccinal strain. These two groups are of special interest as they can be used in tests with improved specificity, and potentially able to differentiate vaccinated and infected individuals. A mutant BCG strain with the DPPD antigen deleted was constructed. The Mb0092 coding sequence was knocked out by homologous recombination. The

sequences flanking the target gene were cloned into a suicide vector. Double crossovers were selected using *sacB.* The knockout genotype was determined by PCR and by Southern blot. This mutant BCG strain can be useful in animal vaccination as it will not interfere in the tuberculosis diagnostic test, when performed using recombinant DPPD. The results show alternatives for the problems related to the use of *M. bovis* BCG as a recombinant vaccine. The auxotrophic complementation system was highly stable, efficient and it is suitable for expressing heterologous antigens in BCG. The identification of proteins present in PPD preparations and the mutant BCG obtained provide the possibility for the development of differential diagnostic test, thus allowing the use of BCG as vaccine also in animals.

Key words: recombinant BCG, auxotrophic complementation, characterization PPD.

SUMÁRIO

1. INTRODUÇÃO GERAL

Mycobacterium bovis BCG, é uma das vacinas mais utilizadas mundialmente, já tendo sido administrada a mais de três bilhões de pessoas. Esta vacina confere uma boa proteção, induzindo tanto resposta imune humoral como celular. Além disso, possui outras qualidades desejáveis para uma vacina, tais como: é um potente adjuvante, segura, estável a temperatura ambiente e de baixo custo de produção, pode ser administrada logo após o nascimento em dose única e por via oral (BLOOM & FINE, 1994).

Os avanços na área da tecnologia do DNA recombinante, o conhecimento do genoma e o desenvolvimento de sistemas de expressão de antígenos heterólogos em BCG, fizeram com que essa bactéria se tornasse um candidate promissor a vetor de vacina recombinante multivalente expressando antígenos heterólogos de outros microrganismos (JACOBS *et al.*, 1987; SNAPPER *et al.*, 1988; HUSSON *et al.*, 1990; MATSUO *et al.*, 1990, DENNEHY & WILLIAMSON, 2005). Vários estudos já demonstraram a viabilidade de BCG em expressar antígenos heterólogos de bactérias, vírus, parasitas, imunomodulares entre outros (O'DONNELL, 1997; OHARA & YAMADA, 2001, DENNEHY & WILLIAMSON, 2005). Em alguns casos, resposta imune protetora contra doenças humanas e animais em modelo murino foi alcançada (ABDELHAK *et al.*, 1995; MATSUMOTO *et al.*, 1998; NASCIMENTO *et al.*, 2000; SEIXAS *et al.*, 2007). Além disso, por ser uma vacina administrada rotineiramente BCG, é o veículo de apresentação de antígenos recombinantes com maior chance de vir a ser licenciado para uso comercial.

No entanto, existem dois problemas quanto a utilização de BCG recombinante como vetor vacinal. O primeiro é a presença de genes que conferem resistência a antibióticos nos vetores utilizados para transformação genética de BCG. O segundo é a limitação da utilização de BCG em animais, principalmente, por comprometer o teste de diagnóstico de tuberculose comumente utilizado. Em virtude disso, novos marcadores seletivos, que possam ser empregados em substituição à resistência por antibióticos, bem como o desenvolvimento de testes de diagnóstico capazes de diferenciar animal infectado de vacinado com BCG se fazem necessários.

A ausência do gene de resistência a antibiótico requer outro marcador de seleção a fim de permitir a seleção das bactérias portadoras do vetor após o processo de transformação. Entre as alternativas está a introdução de genes de resistência à lise por bacteriófagos (DONNELLYWU *et al.*, 1993), ou a metais pesados como o mercúrio (MEISSNER & FALKINHAM, 1984). No entanto, a utilização de mercúrio pode levar a presença de resíduos de metal pesado, o que não é desejável em uma vacina.

A abordagem mais recente para a seleção de recombinantes em BCG é a utilização de marcadores auxotróficos. Um sistema de seleção auxotrófico requer a necessidade do isolamento de cepas de BCG auxotróficas bem definidas, que necessitem para o seu crescimento da suplementação de aminoácidos essenciais não sintetizados em mamíferos (MCADAM *et al.*, 1995; GULERIA *et al.*, 1996; JACKSON *et al.*, 1999; CHAMBERS *et al.*, 2000;PAVELKA *et al.*, 2003; BORSUK *et al.*, 2007). A seleção é realizada através de um vetor de expressão que contenha além do cassete de expressão com o promotor e o gene heterólogo a ser expresso, um gene que complemente a mutação da cepa e restaure a capacidade do BCG de crescer em meio mínimo deficiente do respectivo aminoácido.

A estabilidade dos vetores em BCG recombinante é um fator relevante para o sucesso de uma vacina. Vários trabalhos relatam a baixa estabilidade de vetores plasmidiais quen utilizam gene de resistencia a antibiótico como marcador seletivo (MEDEIROS *et al.*, 2002; DENNEHY & WILLIAMSON, 2005), já que a pressão de seleção exercida pelo antibiótico presente no meio de cultivo deixa de existir quando a vacina é administrada. Vacinação de animais com BCG ainda não é permitida, pois compromete o teste de tuberculina, comumente utilizado para o diagnóstico de tuberculose. O teste de tuberculina não diferencia entre infecção por *M. bovis* e vacinação com BCG ou exposição à micobactérias ambientais. No entanto, este teste é o único validado e utilizado como rotina. Embora existam outros testes moleculares sendo desenvolvidos e avaliados, nenhum apresentou resultados satisfatórios para substituir o teste de tuberculina (Buddle *et al.*, 2001; Dalley *et al.*, 2007; Parra *et al.*, 2007)

A utilização de mutantes auxotróficos como vacinas que não sensibilizam para a tuberculina estão sendo avaliadas (CHAMBERS *et al.*,

2000). Antígenos que estão presentes somente na cepa virulenta, como ESAT6 e CFP10 estão sendo avaliados em testes para diferenciar animais vacinados com BCG e infectados com *M. bovis* (van PINXTEREN *et al.*, 2000; SKJOT *et al.*, 2000; BUDDLE *et al.*, 2006). Testes que detectam IFN gama (IFNγ) após a estimulação de linfócitos em cultura de células sanguíneas tiveram resultados satisfatórios (DALLEY *et al.*, 2007). No entanto, para que BCG possa vir a ser utilizada como vacina em animais, é necessária a disponibilidade de um teste de diagnóstico que seja capaz de diferenciar animais vacinados com BCG daqueles infectados com tuberculose bovina. Além disso, o teste deve ser barato e de fácil execução.

O PPD (Derivado Protéico Purificado) é obtido do crescimento de *Mycobacterium bovis, Mycobacterium tuberculosis* ou *Mycobacterium avium* em meio líquido. A cultura é inativada em autoclave a 121°C por 30 min. O conteúdo protéico é filtrado em malha de aço fina e papel de filtro, e precipitado por meio da adição de ácido tricloracético ou Sulfato de Amônia. A mensuração é realizada através de provas bioquímicas, ajustando-se o conteúdo de nitrogênio protéico conforme os padrões internacionais (DALL'STELLA *et al.*, 2007).

Considerando a ampla utilização deste reagente imunológico para o diagnóstico de tuberculose, é surpreendente que tão pouco é conhecido sobre seus componentes ativos. Existem somente alguns estudos que descrevem algumas proteínas do PPD, tais como o grupo das principais proteínas micobacterianas secretadas: MPB59, MPB64, MPB70, MPB83, CFP10 e ESAT 6 (HUEBNER *et al.*, 1993; KLAUSEN *et al.*, 1994; ROWLAND *et al.*, 1999). Estes antígenos, na forma recombinante, foram avaliados em testes diagnósticos diferencias, sozinhos ou em combinação, mas somente ESAT6 foi capaz de diferenciar animais vacinados com BCG daqueles infectados com tuberculose bovina (BUDDLE *et al.*, 1999; BUDDLE *et al.*, 2001).

Além disso, o teste de tuberculina utilizando o PPD não distingue entre infecção com *M. tuberculosis/bovis* e vacinação com BCG, ou ainda exposição à micobacterias ambientais. Estas reações cruzadas são atribuídas, geralmente à presença no PPD de antígenos comuns às diferentes espécies de micobactérias (DANIEL & JANICKI, 1978; YOUNG, 1992). A identificação dos componentes majoritários de preparações de PPD será muito útil na

identificação de outras proteínas que poderão ser testadas como um PPD recombinante para o diagnóstico diferencial da tuberculose, ou ainda, poderão ser alvos para inativação gênica (*gene knockout*) na cepa vacinal. Dessa forma, a vacina não interferiria com o teste de tuberculina, abrindo caminho para utilização de BCG como vacina contra tuberculose em animais, e também a utilização de BCG recombinante expressando antígenos de outros patógenos.

Neste contexto, as hipóteses deste trabalho foram: o sistema de seleção através da complementação auxotrófica de *M. bovis* BCG Δ*leu*D é mais eficiente do que o sistema de clonagem tradicional que usa resistência a antibiótico como marcador de seleção; e a identificação das proteínas que compõem o PPD vai contribuir para o desenvolvimento de um teste diferencial entre vacinado e infectado por cepa virulenta. Assim foram traçados os seguintes objetivos: (1) Desenvolver e avaliar o sistema de complementação auxotrófica como marcador de seleção em *Mycobacterium bovis* BCG Δ*leu*D baseado na presença do gene *leu*D no vetor plasmidial; (2) Avaliar a estabilidade *in vitro* e *in vivo* dos vetores de complementação auxotrófica em *M. bovis* BCG Δ*leu*D, comparando com *M. bovis* BCG Pasteur; (3) Caracterizar qualitativa e quantitativamente as proteínas componentes do PPD; (4) Desenvolver uma cepa de *M. bovis* BCG Pasteur mutante através da inativação gênica do gene Mb0092 (dppd) por recombinação homóloga.

Inicialmente é apresentada uma revisão bibliográfica sobre a utilização de *Mycobacterium bovis* BCG recombinante (artigo 1). Abordamos as potencialidades e limitações de BCG na expressão de antígenos heterólogos de vírus, bactérias e parasitos, e ainda de imunomoduladores. Discutimos também algumas perspectivas quanto à utilização deste vetor vacinal. O artigo será submetido ao periódico **Vaccine**.

O artigo 2 descreve o desenvolvimento e avaliação de complementação auxotrófica como marcador de seleção para a expressão estável de antígenos heterólogos em *Mycobacterium bovis* BCG Δ*leu*D. Este trabalho foi publicado no periódico **Tuberculosis.**

A identificação das proteínas que compõem preparações de PPD bovino e aviário em amostras do Brasil e da Inglaterra por LC-MS/MS, está descrita no artigo 3, o qual também será submetido ao periódico **Tuberculosis**.

O artigo 4 descreve o desenvolvimento de uma cepa *Mycobacterium bovis* BCG Pasteur mutante pela inativação gênica por recombinação homologa do gene Mb0092 (dppd) e sua importância como vacina em animais. Este será submetido em forma de *Short Communication* para o periódico **FEMS Microbiology Letters**. Os artigos estão compilados na formatação exigida por cada um dos periódicos científicos em que foram ou serão publicados.

2 ARTIGO 1

RECOMBINANT *Mycobacterium bovis* BCG

(Revisão a ser submetida ao periódico *Vaccine*)

RECOMBINANT *Mycobacterium bovis* BCG

Sibele Borsuk[a], Fabiana Kömmling Seixas[a], Reginaldo Bastos[b], Odir A. Dellagostin[a*]

[a] Laboratorio de Biologia Molecular, Centro de Biotecnologia, Universidade Federal de Pelotas, Campus Universitario, Caixa Postal 354, CEP 96010-900 Pelotas, RS, Brazil.

[b] Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164, USA

[*] Corresponding author

Centro de Biotecnologia, Universidade Federal de Pelotas. CP 354, 96010-900, Pelotas, RS, Brazil, Phone: +55 53 32757350. E-mail: odirad@terra.com.br.

ABSTRACT

BCG is an attenuated strain of *Mycobacterium bovis* that has been broadly used as a vaccine against human tuberculosis. This live bacterial vaccine has several advantages; however its efficiency in conferring protection against tuberculosis is a matter of considerable debate. BCG is able to establish a persistent infection and induces both cellular and humoral immune responses. The development of mycobacterial genetic systems to express foreign antigens and the adjuvanticity of BCG are the basis of the potential use of this attenuated mycobacterium as a recombinant vaccine. Over the years, a range of strategies has been developed to allow controlled and stable delivery of viral, bacterial and parasite antigens in BCG. Herein, we review the strategies developed to express heterologous antigens in BCG and the immune response elicited by recombinant BCG. In addition, the use of recombinant BCG as an immunomodulator and the future perspectives of BCG as a recombinant vaccine vector are discussed.

Key words: *Mycobacterium bovis* BCG, recombinant BCG, heterologous expression

INTRODUCTION

The Bacillus Calmette-Guerin (BCG) is a strain of *Mycobacterium bovis* that was empirically attenuated between 1906 and 1920 through several passages on glycerinated bile-potato medium. Subsequent studies inoculating BCG in mice, guinea pigs, calves, rhesus monkeys and chimpanzees demonstrated infectivity but complete attenuation of the strain. During *in vitro* passage, BCG underwent loss and/or rearrangement of several gene complexes that have been identified only recently [1-3]. In 1928, after experimental evaluations, BCG was recommended by the League of Nations as the official vaccine against human tuberculosis. Since then, it remains the only official and commercially available vaccine against tuberculosis (TB) [4]. BCG is currently the world's most widely used vaccine. It has been given to more than 3 billion people since 1921 [5]. BCG offers unique advantages as a vaccine: (1) it is unaffected by maternal antibodies and therefore it can be given at any time after birth; (2) BCG is usually given as a single dose eliciting a long-lasting immunity; (3) it is stable and safe; (4) BCG can be administrated orally; and (5) it is inexpensive to produce when compared to other live vaccines. The extraordinary adjuvant properties of mycobacteria make them an attractive vector for the development of recombinant vaccines [6].

The interest in BCG has increased considerably since the last decade due to the development of genetic systems for expression of foreign antigens in mycobacteria. These systems include the developing of different shuttle-vectors, system to express and secrete heterologous antigens and strategies of mycobacteria transformation. Moreover, technologic advancement in the genomic of mycobacteria improved our understanding of the biology of this slow-growing pathogen and helped the conception of strategies for evaluation of BCG as a vaccine delivery vector [7]. Antigens of bacteria, parasites and viruses have been expressed in this attenuated mycobacterium [8-11]. It has been shown that recombinant BCG (rBCG) elicits both cellular and humoral immune response against the heterologous antigens [12]. Recombinant BCG over expressing antigens of *M. tuberculosis* protects against tuberculosis better than the parental BCG strain [13;14]

This review describes the systems available for heterologous antigen expression and compartmentalization in BCG. In addition, the immune events elicited by recombinant BCG and the use of this attenuated strain as a recombinant immunomodulator are also discussed.

BCG: A VECTOR TO EXPRESS HETEROLOGOUS ANTIGENS

In a pioneer study, Jacobs *et al*. [15] developed an *E. coli*-mycobacteria shuttle "plasmid-cosmid". In their work, a DNA sequence of the mycobacteriophage TM4, isolated from *M. avium*, was ligated to an *E. coli* cosmid and introduced into *E. coli*, *M. smegmatis* and BCG. The recombinant DNA molecule replicated in mycobacteria as a bacteriophage and in *E. coli* as a cosmid. The authors demonstrated not only the possibility of introducing exogenous DNA but also expressing heterologous genes in mycobacteria. However, the TM4-derived shuttle "plasmid-cosmid" was lytic for *M. smegmatis* and BCG, and therefore a new shuttle vector was constructed using the temperate mycobacteriophage L1 [16]. In addition, the aminoglycoside phosphotransferase gene was clone into the temperate shuttle vector conferring stable kanamycin resistance upon transformation [16]. After that, several groups started working and improving the technology for expression of heterologous genes in BCG. Matsuo *et al*. [17] developed a system to express and secrete heterologous antigens from mycobacteria. Stover *et al.* [6] constructed plasmid vectors for expression of heterologous proteins in mycobacteria using the replication elements of the plasmid pAL5000 isolated from *M. fortuitum*. Dellagostin et al. [18] developed an integrative expression system containing two copies of the insertion sequence IS*900* from *M. paratuberculosis* flanking a kanamycin resistance gene in a "suicide" plasmid.

The expression of heterologous genes in BCG can be achieved using either replicating or integrating plasmids. Most of the mycobacterial replicative plasmids are designed using the origin of replication from the pL5000 that allows up to five copies of plasmid per transformants [19]. Therefore, one can expect to express a higher amount of recombinant protein using the replicative plasmids than the integrative vectors. However, it has been demonstrated that

integrative vectors are more genetically stable both *in vitro* and *in vivo* than replicative plasmids [20].

Expression of foreign genes in mycobacteria can be modulated by the promoter used. Several promoters have been widely used in the available *E. coli*-mycobacterium shuttle vectors. The most frequently used are promoters from the heat shock protein genes *hsp60* [6] or *hsp70* [21]. Other promoters used successfully in shuttle vectors include those from the *M. kansasii* (alpha) antigen [17], the *M. paratuberculosis* pAN [22], the *M. tuberculosis* 19 kDa antigen [23] and the *M. fortuitum* β lactamase pBlaF* [24], as well as a compatible promoter from *Streptomyces albus*, GroES/EL1 [12]. Viral antigens have been expressed using *hsp60* and *hsp70* promoter, resulting in high-level of expression [23;25;26] and in some cases conferring protection [27]. The *Sm14* antigen of *Schistosoma mansoni* was expressed in *M. bovis* BCG under the control of the pBlaF* promoter, and this construction was shown to protect mice from cercarial challenge [28].

BCG was originally designed and tested by Calmette and Guerin to be an oral vaccine, and it is important to test recombinant strains of BCG by this route of administration [29]. Hayward *et al.* [30] described an oral vaccination of mice with rBCG expressing the B subunit of *E. coli* heat labile enterotoxin in the mycobacterial cytoplasm, cell wall and in a secreted form. The vaccinated animals developed IgG and IgA antibodies against the *E. coli* antigen, and the strongest response was found in mice vaccinated with rBCG expressing the heterologous antigen in a secreted form. In other study, BCG expressing HIV-1 envelope peptide V3 was tested as an oral vaccine in guinea pigs model. Peripheral blood mononuclear cells from immunized animals showed significant proliferative response [31]. Interestingly, delayed-type hypersensitivity against the HIV peptide was detected even 1.5 year after immunization [31]. Méderlé *et al.* [32] immunized macaques with a single inoculation of three recombinant BCG strains expressing the SIVmac251 *nef*, *gag*, and *env* genes. The animals then received rectal and oral boosting doses of the rBCGs. The authors showed that the mucosal booster dose increased production of IFN-γ and induced production of mucosal IgA.

It has been showed that the dose of BCG is crucial in determining the Th1/Th2 nature of the immune response, with low doses favouring a predominantly cell-mediated Th1 response [33]. Based on that, it is possible to use a specific dose of rBCG to improve the chance to obtain a desirable immune response. A high antibody response was elicited inoculating mice with 10^8 CFU of rBCG expressing the B subunit of human *E. coli* heat labile enterotoxin [30]. Pym et al. [34] reported that 10^6 CFU of rBCG expressing ESAT-6 elicited a strong cellular immunity and, consequently protection against tuberculosis in mouse and guinea pig models.

It has long been known that the adjuvant activity of BCG resides in the mycobacterial cell wall. In order to increase the immunogenicity against foreign antigens expressed in BCG, there may be advantage to use mycobacterial signal sequences to drive the expression to the bacterial wall. Therefore, the MT19 signal sequence, from *M. tuberculosis*, has been used to drive the heterologous antigen to the mycobacterial surface [23;25;27]. Immunization of mice with rBCG expressing the outer surface protein A of *Borrelia burgdorferi* as a membrane-associated lipoprotein resulted in protective antibody response that was 100-1000-fold higher than the response elicited by immunization with rBCG expressing the same antigen expressed cytoplasmically or as a secreted fusion protein [23]. Bastos et al. [27] vaccinated mice with BCG expressing glycoprotein 5 and matrix protein of porcine reproductive and respiratory syndrome virus either in the cytoplasm or on the mycobacterial membrane. In this study, neutralizing antibodies against the virus were only detected in mice inoculated with rBCG expressing the viral antigens on the mycobacterial surface, suggesting an improvement of antigen presenting and induction of humoral immune response. In some cases, it is also interesting to include mycobacterial signal sequences that allow a significant number of recombinant proteins to be secreted from rBCG [30]. The alpha antigen, from *M. kansaii*, has been widely used as a signal sequence to secret foreign antigens from rBCG [17]. Langermann et al. [25] showed protective humoral response in mice vaccinated with rBCG expressing pnemococcal surface protein A on the mycobacterial surface. However, no protection was induced in animals

vaccinated with rBCG expressing the same pneumococcal antigen in a secreted form, suggesting a betterment of immunogenicity.

BCG: A RECOMBINANT VACCINE

Live bacterial vaccines in general require no additional adjuvant component to evoke immune response in several animal models [35]. For BCG, usually a single inoculation is sufficient for induction of immune response and eventually protection. BCG is a delivery vector for heterologous antigens due to its capacity of intracellular replication in antigen presenting cells, such as macrophages and dendritic cells [36]. The induction of immune response against heterologous antigens, following inoculation with rBCG was initially reported by Stover *et al.* [6] and Aldovini & Young [21]. Stover *et al.* [6] tested integrative and multicopy plasmid systems to express heterologous antigens in BCG. Using these two systems, β-galactosidase, tetanic toxin and HIV-1 antigens were expressed in BCG under the control of the mycobacterial *hsp*60 and *hsp*70 gene promoters. Aldovini & Young *et al* [21] utilized a multicopy plasmid system to express HIV-1 proteins in BCG under control of the mycobacteria *hsp*70 gene promoter. Both studies report the development of humoral and cellular immunity against the heterologous antigens following inoculation of the rBCG vaccines in mouse model.

The first evidence of protective immunity elicited by rBCG was demonstrated by Stover *et al.* [23]. In that study, protective humoral immune response was induced in mice inoculated with rBCG expressing *Osp*A antigen from *Borrelia burgdorferi*. Despite of the protection induced by rBCG-*Osp*A in mouse model [23], this recombinant strain failed to elicit protection against Lyme disease in humans [37]. This recombinant vaccine had a safety profile and the volunteers converted positive in the PPD test as expected, but it did not elicit a primary humoral response. Possible explanations to the rBCG-*Osp*A failure in humans are the small amount of *Osp*A production or the loss of the plasmid vector containing the *Osp*A gene [37]. BCG expressing LCR1 antigen of *Leishmania chagasi* induced protective immunity in susceptible mice [38]. BCG expressing glycoprotein 5 and matrix protein of porcine reproductive and

respiratory syndrome virus elicited a degree of protection in swine [27]. Effective protection against cutaneous leishmaniasis was obtained in mouse model using rBCG expressing the leishmania surface proteinase gp63 [39]. Protective immune response against experimental *M. leprae* infection in mice was induced by rBCG over-expressing three components of mycobacterial Ag85 antigen [40]. Immunisation with recombinant BCG expressing the cottontail rabbit papillomavirus (CRPV) L1 gene also provides protection from CRPV challenge [41]. Hamsters immunized with recombinant BCG expressing LipL32 were protected against mortality upon challenge with a lethal inoculum of *L. interrogans* serovar Copenhageni [42].

Recombinant BCG has been used to improve the efficacy of the BCG as a vaccine for tuberculosis, in some cases with better results than the parental vaccine. A recent study with guinea pigs immunized with rBCG30 and challenged with highly virulent *M. tuberculosis* by aerosol, had significantly less organ pathology, significantly fewer organisms in their lungs and spleen, and significantly greater survival than guinea pigs immunized with the parental strain of BCG. This recombinant BCG vaccine was the first vaccine reported to induce greater protective immunity against TB than the standard BCG vaccine in an animal model. A phase I clinical trial with rBCG30 was started in 2004 [43]. In another study, the BCG was equipped with the membrane-perforating listeriolysin (Hly) of *Listeria monocytogenes,* which was shown to improve protection against *M. tuberculosis.* Following aerosol challenge, the Hly-secreting recombinant BCG vaccine was shown to protect significantly better against aerosol infection with *M. tuberculosis* than did the parental BCG strain [44].

Construction of multivalent recombinant BCG is a goal that has been persuaded for many scientific groups. Abomoelak *et al.* [46] expressed in BCG strains a petussis toxin-tetanus toxin. The recombinant strains induce neutralizing antibodies against both toxins in the mouse model. Mederle *et al.* [20] constructed multivalent rBCG strains by using integrative vectors expressing *nef* and *gag* of simian immunodeficiency virus. The strains were highly stable *in vivo* and induced a longer antibody response than non-integrative strains. In addition, the administration of rBCG “cocktails”, containing more than one recombinant strain, has also been shown to elicit immune

response and some level of protection against the heterologous antigens in different animal models [27;47]. It has been demonstrated that expression of heterologous antigens in the mycobacterial cytoplasm, on the mycobacterial surface, or in a secreted form may influence the immune response, and consequently the protective immunity induced against heterologous antigens expressed in BCG. Langerman *et al.* [48] described the development of specific humoral immune response in mice induced by rBCG expressing the *Psp*A antigen from *Streptococcus pneumoniae*. However, only the strain expressing the antigen on the BCG surface and in a secreted form elicited protection.

Despite of the success of heterologous antigen expression and, in some cases, protection induced by rBCG, *in vitro* and *in vivo* instability of the recombinant vaccine has been reported [49]. This instability is reported mainly with replicative vectors that are lost during BCG replication *in vivo* [37]. Vectors that integrate into the BCG genome are more stable [50], however, the disadvantage is the lower expression level of heterologous genes compared to that of multicopy plasmids. Thus, optimization of BCG as a vehicle for live recombinant vaccines requires improved strategies for stable antigen expression. We recently described the construction and evaluation of auxotrophic complementation as a selectable marker for expression of foreign antigens in *Mycobacterium bovis* BCG [45]. The results indicate that this selectable system is stable even during *in vivo* growth, as the selective pressure is maintained.

Table 1, 2, and 3 present a detailed list of heterologous bacterial, parasite, and viral antigens expressed in BCG, as well as the promoter used, route and dose administered, site of expression, immune response elicited and animal model used.

RECOMBINANT BCG: AN IMMUNOMODULATOR

Immunomodulators are substances or live organisms that accelerate, prolong, or enhance the quality of specific immune response to antigens. Live, attenuated, recombinant bacterial vaccines have been largely used to modulate the immune system to respond in a specific profile to a specific antigen [51].

The use of a recombinant BCG expressing IFN-γ (BCG-IFN) resulted in an alteration in the pattern of inflammation and local tissue fibrosis. These results demonstrated that granulomas in the areas of mycobacterial infection are active sites of both inflammation and fibrosis. In addition, local expression of IFN-γ by recombinant BCG results in more efficient bacterial clearance, which is accompanied by a reduction in tissue pathology [52].

The immunomodulator effect of wild type BCG (wtBCG) has been well described [53;54], and recently the expression of cytokines has even improved this effect. This approach has allowed modulation of the immune system to respond with a specific and desired pattern of cytokines [55]. Young *et al.* [56] demonstrated that rBCG secreting IL-2 induced a strong type 1 immune response in mice. It also induced an antibody isotype shift characterizing a type 1 immune response. The authors found that lymphocytes of mice vaccinated with rBCG expressing IL-18 produced significantly less IFN-γ than animals vaccinated with wtBCG. This impaired induction of IFN-γ was correlated to a significantly lower protection against *M. bovis* challenge. In contrast, Biet *et al* [57] demonstrated that rBCG producing IL-18 increased antigen-specific IFN-γ production in mice. Their results showed that the production of IL-18 by rBCG enhanced the immunomudulatory properties of BCG toward a Th1 profile and may be an alternative to treat bladder cancer in human patients. In addition, Luo *et al.* [58] demonstrated that rBCG expressing IL-18 enhanced the Th1 immune response and that such strain of BCG may be used as an agent for bladder cancer immunotherapy.

The knowledge of the mechanisms used by microorganisms and cancer disorders to cause disease, combined with the events that correlate with immune protection, has been used to understand diseases and design more efficient vaccines. However, infections such as tuberculosis and HIV-AIDS among others, are still a challenge for the scientific community. In this context, we are looking forward to seeing a better understanding of the relationship between microorganisms and host in order to support the use of wtBCG and rBCG as immunomodulators to induce protection.

The table 4 present a detailed list of immunomodulators expressed in BCG.

RECOMBINANT BCG: PERSPECTIVES AND CONCLUSIONS

This review described recent progress that has been made towards understanding the recombinant BCG as a vaccine to protect against different diseases. Many foreign antigens have been expressed in BCG and showed to provide adequate immune response and protection. However, a number of factors have been identified as important in the induction of immune response against the foreign antigen, for instance, dose, route, antigen display and stability of the rBCG vaccine.

BCG is a good example of an “old fashion” attenuated vaccine that has been improved by molecular biology techniques. Recombinant BCG has been experimentally tested to elicit immune response and used to induce protection against several infectious diseases. It has been more than 70 years since BCG was officially recommended as a vaccine against TB, and today it still plays an important role in immunization against this disease and it certainly has an undoubted importance as a recombinant vaccine. Over the last 18 years rBCG with enhanced immunostimulatory properties have been genetic engineered and tested. Improvements in vector stability and selection systems have the potential of further improve rBCG not only as a vaccine against tuberculosis, but also against many other diseases, making it a truly multivalent vaccine.

TABLE 1. Bacterial antigens expressed in *Mycobacterium bovis* BCG.

antigen (organism)	promoter	display expression	route	dose (CFU)	immunity	model	reference
LacZ (*E. coli*)	*hsp*60	C	IV,ID,IP	$2x10^6$	HI,CI	mice	[6]
α-galactosidase (*E. coli*)	P_{AN}	C	SC	10^7	HI,IC	mice	[22]
*osp*A (*B. burgdorferi*)	*hsp*60	C,M,S	IP	10^6	HI	mice	[23]
*osp*A (*B. burgdorferi*)	*hsp*60	C	IN,IP	$2x10^8$	HI	mice	[59]
*psp*A (*S. pneumoniae*)	*hsp*60	C,M,S	IP	10^6	HI	mice	[48]
pertusis and tetanic toxin	*ag85a*	C,S	IV,IP	$5x10^6$	HI,CI	mice	[46]
enterotoxin (*E. coli*)	*hsp*60	C,M,S	IP,O	10^8	HI	mice	[30]
*osp*A (*B. burgdorferi*)	*hsp*60	S	ID	10^7	HI	human	[37]
S1 toxin (*B. pertussis*)	*pBla*F*	C	IP	10^6	HI,CI	mice	[60]
Antigens of *M. leprae*	*ag85b*	S	ID	10^7	CI	mice	[61]
ESAT6, 19kDa, 38kDa (*M. tuberculosis*)	*Trrn*	C,S	IV	10^6	HI,CI	mice	[62]
Cholera toxin B subunit	*hsp*60	S	IN	$5x10^6$	HI	mice	[57]
ESAT-6, CFP-10 (*M. tuberculosis*)	*Trrn*	S	SC	10^6	CI	mice guinea pig	[34]
ESAT-6 (*M. tuberculosis*)	esat-6	C,S	SC	10^6	HI,CI	mice	[63]
FC (tetanus toxin fragment)	pBlaF*	C	IP	$5×10^6$	HI, CI	mice	[64]
Ag85B (*M. tuberculosis*)	*S16*	S	IV	10^6	HI,CI	mice	[65]
RD1 antigens	Trrn	S	IV	10^6	CI	mice	[66]
S1 toxin (*B. pertussis*)	P*AN*, hsp60	C	IP	10^6	HI	mice	[67]
30 kDa (*M. tuberculosis*)	*hsp60*	C	ID	10^3	HI, CI	guinea pig	[68]

Ag85B-ESAT6	Hsp60 and a-ss signal sequences	C,S	SC	10^6	HI,CI	mice	[69]
LipL32 (*L. interrogans*)	PAN, hsp60	C,M	IP	10^6	HI	mice	[70]
Esat-6 (*M. tuberculosis*)	hsp60	S	SC	10^6	HI, CI	mice	[71]
Ag85A (*M. tuberculosis)*	pBBN vector (Ag85A-HA)		ID	$2x10^6$	CI	monkey	[72]
EmII-3, Em14-3-3 (*E. multilocularis)*	-	-	SC, IN	-	HI, CI	mice	[73]
Ag85B, ESAT-6 (*M. tuberculosis*)	*hsp60*	S	SC	$5×10^6$	HI, CI	mice	[74]
LipL32 (*L. interrogans*)	*PAN, hsp60, 18kDa*	C, S	IP	10^6	HI, CI	hamster	[42]
S1 (pertussis toxin)	*pBla*F*	C	IP	10^6	CI	mice	[75]
ag85B–mpt64, mtb8.4 (*M. tuberculosis*)	pMV261 (hsp60)	S	SC	5×10^6	HI, CI	mice	[76]

hsp: heat shock protein. C: cytoplasm. M: membrane surface. S: secreted. IV: intravenous injection. ID: intradermal injection. IP: intraperitoneal injection. SC: subcutaneous injection. IN: intranasal. O: oral. IM: intramuscular CFU: colony-forming unit. HI: humoral immunity. CI: cellular immunity.

TABLE 2. Parasite antigens expressed in *Mycobacterium bovis* BCG.

antigen (organism)	promoter	display expression	route	dose (CFU)	immunity	model	reference
gp63 (*Leishmania spp*)	*hsp*60	C	SC,IV	10^6	HI	mice	[39]
S-transferase (*S. mansoni*)	*hsp*60	C,S	IV,IP,SC	10^6	HI	mice	[77]
*gra*1 (*T. gondii*)	85A	C,S	IP	$5x10^6$	HI,CI	mice and lamb	[78]
*lcr*1 (*L. chagasi*)	*hsp*60	C	IP,SC	10^6	HI,CI	mice	[38]
Surface antigens (*P. falciparum*)	*hsp*70	C	SC	10^7	HI,CI	mice	[79]
Sj26GST (*S. japonicum)*	*hsp70*	C	-	-	HI,CI	mice	[80]
Sm14 (S. mansoni)	*pBlaF**	M	IP	10^6	HI,CI	mice	[28]
msp1A (A. marginale)	*pBla*F*, 18kDa,	C	IP	10^6	HI,CI	mice	[81]
Malarial epitopes F2R(II) EBA and (NANP)3	*PAL500*	-	IP	10^6	HI,CI	mice	[82]
ROP2 (*T. gondii*)	*hsp60*	C	-	-	HI,CI	mice	[83]
GRA4 or SAG2 (*T. gondii*)	-	-	IP	-	HI	mice	[84]

hsp: heat shock protein. C: cytoplasm. M: membrane surface. S: secreted. IV: intravenous injection. ID: intradermal injection. IP: intraperitoneal injection. SC: subcutaneous injection. IN: intranasal. O: oral. IM: intramuscular CFU: colony-forming unit. HI: humoral immunity. CI: cellular immunity.

TABLE 3. Viral antigens expressed in *Mycobacterium bovis* BCG.

antigen (organism)	promoter	site	route	dose (CFU)	immunity	model	reference
gag, *env,* and p*ol* (HIV-1)	*hsp*70	C	ID	$5x10^6$	HI,CI	mice	[21]
nef (HIV-1)	*groES*	C	SC	10^7	CI	mice	[85]
gag (SIV)	*hsp70*	C	ID	10^8	CI	macaque	[86]
nef (SIV)	P_{AN}	C	SC	10^7	HI,CI	mice	[87]
L1 e E7 (papilomavirus)	*hsp*70	C	IV,SC,IN	10^6	HI,CI	mice	[88]
nef, g*ag,* and e*nv* (SIV)	*hsp*70	C	SC,IV	10^8	HI,CI	macaque	[47]
Epitopes of rabies virus	18kDa, *hsp*60	C	IP	10^6	HI	mice	[89]
GP5 and M protein (PRRSV)	*hsp*60	C,M	IP	10^8	HI,CI	mice	[90]
V3 (HIV-1)	*hsp*60	-	SC,O	320 mg	HI,CI	guinea pig	[31]
Nef, gag, env (SIV)	P_{AN}	C	ID,O,R	$5x10^8$	CI	monkey	[32]
CTL epitope (HCV)	-	S	IP	$2x10^7$	CI	mice	[91]
GP5 and M protein (PRRSV)	hsp60	M	IM	10^8	HI,CI	swine	[27]
S genes (Hepatitis virus)	P_{AN}	C	IP	10^7	HI	mice	[92]
Gag (SIV)	hsp60	C	ID	10^7	CI	macaque	[93]
p24 Gag (HIV-1)	hsp60	C	ID	10^7	HI,CI	mice	[26]
Env V3 (HIV)	*hsp*60	-	-	-	-	macaque	[94]
I1 (CRPV)	*mtrA*	S	SC	10^7	HI, CI	rabbit	[41]
p27Gag (SIV)	hsp60	C	ID, O	80-160mg	HI, CI	guinea pig	[95]

VP6 (rotavirus)	*hsp60*	C,M,S	IP	-	HI,CI	mice	[96]
Glycoprotein Si (Infectious bronchitis)	*hsp70*	M	IP	10^6	-	mice	[97]
Env (HIV)	-	C,M,S	-	-	CI	mice	[98]
papillomavirus (CRPV)	*mtrA*	S	SC	10^7	-	rabbit	[99]

hsp: heat shock protein. C: cytoplasm. M: membrane surface. S: secreted. IV: intravenous injection. ID: intradermal injection. IP: intraperitoneal injection. SC: subcutaneous injection. IN: intranasal. O: oral. IM: intramuscular. R: rectal. CFU: colony-forming unit. HI: humoral immunity. CI: cellular immunity. HIV: human immunodeficient virus. SIV: simian immunodeficient virus PRRS: Porcine reproductive and respiratory syndrome. HCV: hepatitis C virus.

TABLE 4. Cytokines expressed in *Mycobacterium bovis* BCG.

cytokine	Animal Model	**immunological effect**	**reference**
IL-2	mice	efficient cytotoxicity, cytokines such as IL-12, tumor necrosis factor and interferon (IFN)-gamma	[100]
IL-2 andIL-18	mice	T helper 1-type immune response	[101]
IL-18	mice	IFN-γ production	[57]
IL-18	mice	T helper 1-type immune response	[58]
IL-12	mice	-	[102]
IFN gamma	mice	up-regulated expression of MHC class I molecules	[103]
IL-18 and IL-5	mice	Th2 response	[104]

REFERENCES

[1] Behr MA, Wilson MA, Gill WP, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999 May 28;284(5419):1520-3.

[2] Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J Bacteriol 1996 Mar;178(5):1274-82.

[3] Brosch R, Pym AS, Gordon SV, Cole ST. The evolution of mycobacterial pathogenicity: clues from comparative genomics. Trends Microbiol 2001 Sep;9(9):452-8.

[4] Lugosi L. Theoretical and methodological aspects of BCG vaccine from the discovery of Calmette and Guerin to molecular biology. A review. Tuber Lung Dis 1992 Oct;73(5):252-61.

[5] Bloom BR, Fine PEM. The BCG experience: implications for future vaccines against tuberculosis. In: Bloom BR, editor. Tuberculosis: Pathogenesis, Protection, and Control.Washington, DC, ASM Press, 1994: p. 531-58.

[6] Stover CK, de IC, V, Fuerst TR, et al. New use of BCG for recombinant vaccines. Nature 1991 Jun 6;351(6326):456-60.

[7] Philipp WJ, Schwartz DC, Telenti A, Cole ST. Mycobacterial genome structure. Electrophoresis 1998 Apr;19(4):573-6.

[8] Chan J, Kaufmann SHE. Immune mechanism of protection. In: Bloom BR, editor. Tuberculosis: Pathogenesis, Protection, and Control.Washington, DC, ASM Press, 1994: p. 398-415.

[9] Bloom BR, Modlin RL, Salgame P. Stigma variations: observations on suppressor T cells and leprosy. Annu Rev Immunol 1992;10:453-88.

[10] Mogues T, Goodrich ME, Ryan L, LaCourse R, North RJ. The relative importance of T cell subsets in immunity and immunopathology of airborne *Mycobacterium tuberculosis* infection in mice. J Exp Med 2001 Feb 5;193(3):271-80.

[11] Agger EM, Andersen P. A novel TB vaccine; towards a strategy based on our understanding of BCG failure. Vaccine 2002 Nov 22;21(1-2):7-14.

[12] Dennehy M, Williamson AL. Factors influencing the immune response to foreign antigen expressed in recombinant BCG vaccines. Vaccine 2005 Jan 26;23(10):1209-24.

[13] Castanon-Arreola M, Lopez-Vidal Y, Espitia-Pinzon C, Hernandez-Pando R. A new vaccine against tuberculosis shows greater protection in a mouse model with progressive pulmonary tuberculosis. Tuberculosis 2005 Jan;85(1-2):115-26.

[14] Horwitz MA. Recombinant BCG expressing *Mycobacterium tuberculosis* major extracellular proteins. Microbes Infect 2005 May;7(5-6):947-54.

[15] Jacobs WR, Jr., Tuckman M, Bloom BR. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature 1987 Jun 11;327(6122):532-5.

[16] Snapper SB, Lugosi L, Jekkel A, et al. Lysogeny and transformation in mycobacteria: stable expression of foreign genes. Proc Natl Acad Sci U S A 1988 Sep;85(18):6987-91.

[17] Matsuo K, Yamaguchi R, Yamazaki A, et al. Establishment of a foreign antigen secretion system in mycobacteria. Infect Immun 1990 Dec;58(12):4049-54.

[18] Dellagostin OA, Esposito G, Eales LJ, Dale JW, McFadden J. Activity of mycobacterial promoters during intracellular and extracellular growth. Microbiology 1995 Aug;141 (Pt 8):1785-92.

[19] Labidi A, David HL, Roulland-Dussoix D. Restriction endonuclease mapping and cloning of *Mycobacterium fortuitum* var. fortuitum plasmid pAL5000. Ann Inst Pasteur Microbiol 1985 Sep;136B(2):209-15.

[20] Mederle I, Bourguin I, Ensergueix D, et al. Plasmidic versus insertional cloning of heterologous genes in *Mycobacterium bovis* BCG: impact on in vivo antigen persistence and immune responses. Infect Immun 2002 Jan;70(1):303-14.

[21] Aldovini A, Young RA. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature 1991 Jun 6;351(6326):479-82.

[22] Murray A, Winter N, Lagranderie M, et al. 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 1992 Nov;6(22):3331-42.

[23] Stover CK, Bansal GP, Hanson MS, et al. Protective immunity elicited by recombinant bacille Calmette-Guerin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. J Exp Med 1993 Jul 1;178(1):197-209.

[24] Timm J, Lim EM, Gicquel B. *Escherichia coli*-mycobacteria shuttle vectors for operon and gene fusions to *lacZ*: the pJEM series. J Bacteriol 1994 Nov;176(21):6749-53.

[25] Langermann S, Palaszynski S, Sadziene A, Stover CK, Koenig S. Systemic and mucosal immunity induced by BCG vector expressing outer-surface protein A of *Borrelia burgdorferi*. Nature 1994 Dec 8;372(6506):552-5.

[26] Kanekiyo M, Matsuo K, Hamatake M, et al. Mycobacterial codon optimization enhances antigen expression and virus-specific immune responses in recombinant *Mycobacterium bovis* bacille Calmette-Guerin expressing human immunodeficiency virus type 1 Gag. J Virol 2005 Jul;79(14):8716-23.

[27] Bastos RG, Dellagostin OA, Barletta RG, et al. Immune response of pigs inoculated with *Mycobacterium bovis* BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. 2003.

[28] Varaldo PB, Leite LC, Dias WO, et al. Recombinant *Mycobacterium bovis* BCG expressing the Sm14 antigen of *Schistosoma mansoni* protects mice from cercarial challenge. Infect Immun 2004 Jun;72(6):3336-43.

[29] Barletta RG, Snapper B, Cirillo JD, et al. Recombinant BCG as a candidate oral vaccine vector. Res Microbiol 1990 Sep;141(7-8):931-9.

[30] Hayward CM, O'Gaora P, Young DB, et al. Construction and murine immunogenicity of recombinant Bacille Calmette Guerin vaccines expressing the B subunit of *Escherichia coli* heat labile enterotoxin. Vaccine 1999 Mar 5;17(9-10):1272-81.

[31] Kawahara M, Hashimoto A, Toida I, Honda M. Oral recombinant *Mycobacterium bovis* bacillus Calmette-Guerin expressing HIV-1 antigens as a freeze-dried vaccine induces long-term, HIV-specific mucosal and systemic immunity. Clin Immunol 2002 Dec;105(3):326-31.

[32] Mederle I, Le Grand R, Vaslin B, et al. 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 2003 Oct 1;21(27-30):4153-66.

[33] Power CA, Wei G, Bretscher PA. Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. Infect Immun 1998 Dec;66(12):5743-50.

[34] Pym AS, Brodin P, Majlessi L, et al. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat Med 2003 May;9(5):533-9.

[35] Roland KL, Tinge SA, Killeen KP, Kochi SK. Recent advances in the development of live, attenuated bacterial vectors. Curr Opin Mol Ther 2005 Feb;7(1):62-72.

[36] Nasser EA, Kaufmann SH. Improved protection by recombinant BCG. Microbes Infect 2005 May;7(5-6):939-46.

[37] Edelman R, Palmer K, Russ KG, 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 Feb 26;17(7-8):904-14.

[38] Streit JA, Recker TJ, Donelson JE, Wilson ME. BCG expressing LCR1 of *Leishmania chagasi* induces protective immunity in susceptible mice. Exp Parasitol 2000 Jan;94(1):33-41.

[39] Connell ND, Medina-Acosta E, McMaster WR, Bloom BR, Russell DG. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. Proc Natl Acad Sci U S A 1993 Dec 15;90(24):11473-7.

[40] Ohara N, Yamada T. Recombinant BCG vaccines. Vaccine 2001 Jul 20;19(30):4089-98.

[41] Govan VA, Christensen ND, Berkower C, Jacobs WR, Jr., Williamson AL. Immunisation with recombinant BCG expressing the cottontail rabbit papillomavirus (CRPV) L1 gene provides protection from CRPV challenge. Vaccine 2006 Mar 15;24(12):2087-93.

[42] Seixas FK, Silva EF, Hartwig DD, et al. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of Leptospira interrogans protects hamsters from challenge. Vaccine 2007.

[43] Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. Enhancing the protective efficacy of *Mycobacterium bovis* BCG vaccination against tuberculosis by boosting with the *Mycobacterium tuberculosis* major secretory protein. Infect Immun 2005 Aug;73(8):4676-83.

[44] Grode L, Seiler P, Baumann S, et al. Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest 2005 Sep;115(9):2472-9.

[45] Borsuk S, Mendum TA, Fagundes MQ, et al. Auxotrophic complementation as a selectable marker for stable expression of foreign antigens in *Mycobacterium bovis* BCG. Tuberculosis 2007 Nov;87(6):474-80.

[46] Abomoelak B, Huygen K, Kremer L, Turneer M, Locht C. Humoral and cellular immune responses in mice immunized with recombinant *Mycobacterium bovis* Bacillus Calmette-Guerin producing a pertussis toxin-tetanus toxin hybrid protein. Infect Immun 1999 Oct;67(10):5100-5.

[47] Leung NJ, Aldovini A, Young R, et al. The kinetics of specific immune responses in rhesus monkeys inoculated with live recombinant BCG expressing SIV Gag, Pol, Env, and Nef proteins. Virology 2000 Mar 1;268(1):94-103.

[48] Langermann S, Palaszynski SR, Burlein JE, et al. Protective humoral response against pneumococcal infection in mice elicited by recombinant bacille Calmette-Guerin vaccines expressing pneumococcal surface protein A. J Exp Med 1994 Dec 1;180(6):2277-86.

[49] Medeiros MA, Dellagostin OA, Armoa GR, et al. Comparative evaluation of *Mycobacterium vaccae* as a surrogate cloning host for use in the study of mycobacterial genetics. Microbiology 2002 Jul;148(Pt 7):1999-2009.

[50] Dellagostin OA, Wall S, Norman E, O'Shaughnessy T, Dale JW, McFadden J. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol Microbiol 1993 Dec;10(5):983-93.

[51] Murray PJ, Aldovini A, Young RA. Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines. Proc Natl Acad Sci U S A 1996 Jan 23;93(2):934-9.

[52] Wangoo A, Brown IN, Marshall BG, Cook HT, Young DB, Shaw RJ. Bacille Calmette-Guerin (BCG)-associated inflammation and fibrosis: modulation by recombinant BCG expressing interferon-gamma (IFN-gamma). Clin Exp Immunol 2000 Jan;119(1):92-8.

[53] Kavoussi LR, Torrence RJ, Gillen DP, et al. Results of 6 weekly intravesical bacillus Calmette-Guerin instillations on the treatment of superficial bladder tumors. J Urol 1988 May;139(5):935-40.

[54] Alexandroff AB, Jackson AM, O'Donnell MA, James K. BCG immunotherapy of bladder cancer: 20 years on. Lancet 1999 May 15;353(9165):1689-94.

[55] O'Donnell MA, Aldovini A, Duda RB, et al. Recombinant *Mycobacterium bovis* BCG secreting functional interleukin-2 enhances gamma interferon production by splenocytes. Infect Immun 1994 Jun;62(6):2508-14.

[56] Young SL, O'Donnell MA, Buchan GS. IL-2-secreting recombinant bacillus Calmette Guerin can overcome a Type 2 immune response and corticosteroid-induced immunosuppression to elicit a Type 1 immune response. Int Immunol 2002 Jul;14(7):793-800.

[57] Biet F, Kremer L, Wolowczuk I, Delacre M, Locht C. *Mycobacterium bovis* BCG producing interleukin-18 increases antigen-specific gamma interferon production in mice. Infect Immun 2002 Dec;70(12):6549-57.

[58] Luo Y, Yamada H, Chen X, et al. Recombinant *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) expressing mouse IL-18 augments Th1 immunity and macrophage cytotoxicity. Clin Exp Immunol 2004 Jul;137(1):24-34.

[59] Lagranderie M, Murray A, Gicquel B, Leclerc C, Gheorghiu M. Oral immunization with recombinant BCG induces cellular and humoral immune responses against the foreign antigen. Vaccine 1993 Oct;11(13):1283-90.

[60] Nascimento IP, Dias WO, Mazzantini RP, et al. Recombinant *Mycobacterium bovis* BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live *Bordetella pertussis* in mice. Infect Immun 2000 Sep;68(9):4877-83.

[61] Ohara N, Matsuoka M, Nomaguchi H, Naito M, Yamada T. Protective responses against experimental *Mycobacterium leprae* infection in mice induced by recombinant Bacillus Calmette-Guerin over-producing three putative protective antigen candidates. Vaccine 2001 Feb 28;19(15-16):1906-10.

[62] Rao V, Dhar N, Tyagi AK. Modulation of host immune responses by overexpression of immunodominant antigens of *Mycobacterium tuberculosis* in bacille Calmette-Guerin. Scand J Immunol 2003 Oct;58(4):449-61.

[63] Bao L, Chen W, Zhang H, Wang X. Virulence, immunogenicity, and protective efficacy of two recombinant *Mycobacterium bovis* bacillus Calmette-Guerin strains expressing the antigen ESAT-6 from *Mycobacterium tuberculosis*. Infect Immun 2003 Apr;71(4):1656-61.

[64] Mazzantini RP, Miyaji EN, Dias WO, et al. Adjuvant activity of *Mycobacterium bovis* BCG expressing CRM197 on the immune response induced by BCG expressing tetanus toxin fragment C. Vaccine 2004 Jan 26;22(5-6):740-6.

[65] Dhar N, Rao V, Tyagi AK. Immunogenicity of recombinant BCG vaccine strains overexpressing components of the antigen 85 complex of *Mycobacterium tuberculosis*. Med Microbiol Immunol 2004 Feb;193(1):19-25.

[66] Demangel C, Garnier T, Rosenkrands I, Cole ST. Differential effects of prior exposure to environmental mycobacteria on vaccination with *Mycobacterium bovis* BCG or a recombinant BCG strain expressing RD1 antigens. Infect Immun 2005 Apr;73(4):2190-6.

[67] Medeiros MA, Armoa GR, Dellagostin OA, McIntosh D. Induction of humoral immunity in response to immunization with recombinant *Mycobacterium bovis* BCG expressing the S1 subunit of *Bordetella pertussis* toxin. Can J Microbiol 2005 Dec;51(12):1015-20.

[68] Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. A novel live recombinant mycobacterial vaccine against bovine tuberculosis more potent than BCG. Vaccine 2006 Mar 6;24(10):1593-600.

[69] Shi C, Wang X, Zhang H, Xu Z, Li Y, Yuan L. Immune responses and protective efficacy induced by 85B antigen and early secreted antigenic target-6 kDa antigen fusion protein secreted by recombinant bacille Calmette-Guerin. Acta Biochim Biophys Sin (Shanghai) 2007 Apr;39(4):290-6.

[70] Seixas FK, Fernandes CH, Hartwig DD, Conceição FR, Aleixo JA, Dellagostin OA. Evaluation of different ways of presenting LipL32 to the immune system with the aim of developing a recombinant vaccine against leptospirosis. Can J Microbiol 2007 Apr;53(4):472-9.

[71] Wang LM, Shi CH, Fan XL, Xue Y, Bai YL, Xu ZK. Expression and immunogenicity of recombinant *Mycobacterium bovis* Bacillus

Calmette-Guerin strains secreting the antigen ESAT-6 from *Mycobacterium tuberculosis* in mice. Chin Med J (Engl) 2007 Jul 20;120(14):1220-5.

[72] Sugawara I, Li Z, Sun L, Udagawa T, Taniyama T. Recombinant BCG Tokyo (Ag85A) protects cynomolgus monkeys (*Macaca fascicularis*) infected with H37Rv Mycobacterium tuberculosis. Tuberculosis 2007 Nov;87(6):518-25.

[73] Li WG, Wang H, Zhu YM. [Changes of cytokines of splenocytes in mice immunized by mix recombinant BCG-EmII/3 and BCG-Em14-3-3 vaccine of Em.]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2007 Oct;23(10):911-3.

[74] Xu Y, Zhu B, Wang Q, et al. Recombinant BCG coexpressing Ag85B, ESAT-6 and mouse-IFN-gamma confers effective protection against *Mycobacterium tuberculosis* in C57BL/6 mice. FEMS Immunol Med Microbiol 2007 Dec;51(3):480-7.

[75] Nascimento IP, Dias WO, Quintilio W, et al. Neonatal immunization with a single dose of recombinant BCG expressing subunit S1 from pertussis toxin induces complete protection against *Bordetella pertussis* intracerebral challenge. Microbes Infect 2007 Nov 20.

[76] Y.Q.Qie, J.L.Wang, B.D.Zhu, et al. Evaluation of a new Recombinant BCG which Contains Mycobacterial Antigen ag85B–mpt64190–198–mtb8.4 in C57.BL6 Mice. Scandinavian Journal of Immunology 2008;67:133-9.

[77] Kremer L, Dupre L, Riveau G, Capron A, Locht C. Systemic and mucosal immune responses after intranasal administration of recombinant *Mycobacterium bovis* bacillus Calmette-Guerin expressing glutathione S-transferase from Schistosoma haematobium. Infect Immun 1998 Dec;66(12):5669-76.

[78] Supply P, Sutton P, Coughlan SN, et al. Immunogenicity of recombinant BCG producing the GRA1 antigen from *Toxoplasma gondii*. Vaccine 1999 Feb 26;17(7-8):705-14.

[79] Zheng C, Xie P, Chen Y. Immune response induced by recombinant BCG expressing merozoite surface antigen 2 from *Plasmodium falciparum*. Vaccine 2001 Dec 12;20(5-6):914-9.

[80] Dai W, Gao H, Huang H, Yuan Y, Hu J, Huangfu Y. Comparative study on the immunogenicity between recombinant MS-Sj26GST vaccine and recombinant BCG-Sj26GST vaccine in *Schistosoma japonicum*. J Huazhong Univ Sci Technolog Med Sci 2003;23(3):213-5, 218.

[81] Michelon A, Conceição FR, Binsfeld PC, et al. Immunogenicity of *Mycobacterium bovis* BCG expressing *Anaplasma marginale* MSP1a antigen. Vaccine 2006 Sep 11;24(37-39):6332-9.

[82] Rapeah S, Norazmi MN. Immunogenicity of a recombinant *Mycobacterium bovis* bacille Calmette-Guerin expressing malarial and tuberculosis epitopes. Vaccine 2006 Apr 24;24(17):3646-53.

[83] Wang H, Liu Q, Liu K, et al. Immune response induced by recombinant *Mycobacterium bovis* BCG expressing ROP2 gene of *Toxoplasma gondii*. Parasitol Int 2007 May 10.

[84] Li JH, Wu ST, Weng YB, et al. [Study on immuno-effect with GRA4 or SAG2 gene recombinant BCG vaccine of *Toxoplasma gondii*. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 2007 Jun;25(3):217-21.

[85] Winter N, Lagranderie M, Rauzier J, et al. Expression of heterologous genes in *Mycobacterium bovis* BCG: induction of a cellular response against HIV-1 Nef protein. Gene 1991 Dec 20;109(1):47-54.

[86] Yasutomi Y, Koenig S, Haun SS, et al. Immunization with recombinant BCG-SIV elicits SIV-specific cytotoxic T lymphocytes in rhesus monkeys. J Immunol 1993 Apr 1;150(7):3101-7.

[87] Winter N, Lagranderie M, Gangloff S, Leclerc C, Gheorghiu M, Gicquel B. Recombinant BCG strains expressing the SIVmac251nef gene induce proliferative and CTL responses against nef synthetic peptides in mice. Vaccine 1995 Apr;13(5):471-8.

[88] Jabbar IA, Fernando GJ, Saunders N, et al. Immune responses induced by BCG recombinant for human papillomavirus L1 and E7 proteins. Vaccine 2000 May 8;18(22):2444-53.

[89] da Cruz FW, McBride AJ, Conceicao FR, Dale JW, McFadden J, Dellagostin OA. Expression of the B-cell and T-cell epitopes of the rabies virus nucleoprotein in *Mycobacterium bovis* BCG and induction of an humoral response in mice. Vaccine 2001 Dec 12;20(5-6):731-6.

[90] Bastos RG, Dellagostin OA, Barletta RG, Doster AR, Nelson E, Osorio FA. Construction and immunogenicity of recombinant *Mycobacterium bovis* BCG expressing GP5 and M protein of porcine reproductive respiratory syndrome virus. Vaccine 2002 Nov 22;21(1-2):21-9.

[91] Uno-Furuta S, Matsuo K, Tamaki S, et al. Immunization with recombinant Calmette-Guerin bacillus (BCG)-hepatitis C virus (HCV)

elicits HCV-specific cytotoxic T lymphocytes in mice. Vaccine 2003 Jul 4;21(23):3149-56.

[92] Rezende CA, De Moraes MT, De SM, McIntoch D, Armoa GR. Humoral response and genetic stability of recombinant BCG expressing hepatitis B surface antigens. J Virol Methods 2005 Apr;125(1):1-9.

[93] Ami Y, Izumi Y, Matsuo K, et al. Priming-boosting vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guerin and a nonreplicating vaccinia virus recombinant leads to long-lasting and effective immunity. J Virol 2005 Oct;79(20):12871-9.

[94] Someya K, Cecilia D, Ami Y, et al. Vaccination of rhesus macaques with recombinant *Mycobacterium bovis* bacillus Calmette-Guerin Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not a heterologous V3 motif. J Virol 2005 Feb;79(3):1452-62.

[95] Kawahara M, Matsuo K, Honda M. Intradermal and oral immunization with recombinant *Mycobacterium bovis* BCG expressing the simian immunodeficiency virus Gag protein induces long-lasting, antigen-specific immune responses in guinea pigs. Clin Immunol 2006 Apr;119(1):67-78.

[96] Dennehy M, Bourn W, Steele D, Williamson AL. Evaluation of recombinant BCG expressing rotavirus VP6 as an anti-rotavirus vaccine. Vaccine 2007 May 4;25(18):3646-57.

[97] Bai JN, Bian YQ, Zhao BH. Construction of recombinant BCG bearing S1 glycoprotein of nephropathogenic IBV and study on its immunogenicity on chickens. Wei Sheng Wu Xue Bao 2007 Apr;47(2):324-8.

[98] Yu JS, Peacock JW, Jacobs WR, Jr., et al. Recombinant bacillus Calmette-Guerin elicits HIV-1 envelope-specific T lymphocytes at mucosal sites. Clin Vaccine Immunol 2007 May 16.

[99] Govan VA, Williamson AL. Rabbits immunised with recombinant BCG expressing the cottontail rabbit papillomavirus (CRPV) L2E7E2 genes induces regression of established papillomas. Virus Res 2007 Jul;127(1):43-8.

[100] Matsumoto S, Yukitake H, Kanbara H, Yamada H, Kitamura A, Yamada T. *Mycobacterium bovis* bacillus calmette-guerin induces protective immunity against infection by *Plasmodium yoelii* at blood-stage depending on shifting immunity toward Th1 type and inducing

protective IgG2a after the parasite infection. Vaccine 2000 Nov 22;19(7-8):779-87.

[101] Young S, O'Donnell M, Lockhart E, et al. Manipulation of immune responses to *Mycobacterium bovis* by vaccination with IL-2- and IL-18-secreting recombinant bacillus Calmette Guerin. Immunol Cell Biol 2002 Jun;80(3):209-15.

[102] Lee CF, Chang SY, Hsieh DS, Yu DS. Immunotherapy for bladder cancer using recombinant bacillus Calmette-Guerin DNA vaccines and interleukin-12 DNA vaccine. J Urol 2004 Mar;171(3):1343-7.

[103] Arnold J, de Boer EC, O'Donnell MA, Bohle A, Brandau S. Immunotherapy of experimental bladder cancer with recombinant BCG expressing interferon-gamma. J Immunother (1997) 2004 Mar;27(2):116-23.

[104] Biet F, Duez C, Kremer L, et al. Recombinant *Mycobacterium bovis* BCG producing IL-18 reduces IL-5 production and bronchoalveolar eosinophilia induced by an allergic reaction. Allergy 2005 Aug;60(8):1065-72.

3 ARTIGO 2

AUXOTROPHIC COMPLEMENTATION AS A SELECTABLE MARKER FOR STABLE EXPRESSION OF FOREIGN ANTIGENS IN *Mycobacterium bovis* BCG

(Artigo publicado no periódico *Tuberculosis*)

AUXOTROPHIC COMPLEMENTATION AS A SELECTABLE MARKER FOR STABLE EXPRESSION OF FOREIGN ANTIGENS IN *Mycobacterium bovis* BCG

Sibele Borsuk[a], Tom A. Mendum[b], Michel Quevedo Fagundes[a], Marcelo Michelon[a], Cristina Wetzel Cunha[a], Johnjoe McFadden[b], Odir Antônio Dellagostin[a,*]

[a] *Centro de Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil,*

[b] *School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, UK.*

[*] Corresponding author

Centro de Biotecnologia, Universidade Federal de Pelotas. CP 354, 96010-900, Pelotas, RS, Brazil, Phone: +55 53 32757350. E-mail: odirad@terra.com.br.

ABSTRACT

Mycobacterium bovis BCG has the potential to be an effective live vector for multivalent vaccines. However, most mycobacterial cloning vectors rely on antibiotic resistance genes as selectable markers, which would be undesirable in any practical vaccine. Here we report the use of auxotrophic complementation as a selectable marker that would be suitable for use in a recombinant vaccine. A BCG auxotrophic for the amino acid leucine was constructed by knocking out the *leuD* gene by unmarked homologous recombination. Expression of *leuD* on a plasmid not only allowed complementation, but also acted as a selectable marker. Removal of the kanamycin resistance gene, which remained necessary for plasmid manipulations in *E. coli*, was accomplished by two different methods: restriction enzyme digestion followed by re-ligation before BCG transformation, or by Cre-*loxP in vitro* recombination mediated by the bacteriophage P1 Cre recombinase. Stability of the plasmid was evaluated during *in vitro* and *in vivo* growth of the recombinant BCG in comparison to selection by antibiotic resistance. The new system was highly stable even during *in vivo* growth, as the selective pressure is maintained, whereas the conventional vector was unstable in the absence of selective pressure. This new system will now allow the construction of potential recombinante vaccine strains using stable multicopy plasmid vectors without the inclusion of antibiotic resistance markers.

Keywords: recombinant BCG; auxotrophic complementation; foreign antigens

1. INTRODUCTION

The live attenuated *Mycobacterium bovis* strain Bacille Calmette-Guerin (BCG) is widely used as a vaccine against tuberculosis. It also has many properties that make it one of the most attractive live vectors for the development of recombinant vaccines against other diseases, for example: it is unaffected by maternal antibodies and therefore it can be given at any time after birth; it is usually given as a single dose eliciting both humoral and cell-mediated immune responses; it is stable and safe; it can be administrated orally; and it is inexpensive to produce when compared to other live vaccines.[1,2] Development of expression vectors for mycobacteria has allowed the construction of recombinant BCG (rBCG) that express a variety of foreign antigens that, in some instances, induced protective immune responses in animal models.[3-6] Recombinant BCG has also been used to express *M. tuberculosis* proteins for developing an improved vaccine against tuberculosis.[7] Expression of the *M. tuberculosis* 30 kDa major secretory protein in BCG resulted in greater protective immunity than wild type BCG against human[8] and bovine tuberculosis.[9]

A large number of *E. coli*-mycobacterium shuttle vectors have been developed for the transfer of foreign genes into BCG. These are maintained in mycobacteria either episomally or by integrating into the mycobacterial genome. Most episomal plasmids have been developed by combining a region of the mycobacterial replicon of the *M. fortuitum* pAL5000 plasmid[10] with an *E. coli* cloning vector and a kanamycin-resistance gene.[11] These shuttle vectors replicate in mycobacteria at about five copies per genome.[1] However, expression systems for heterologous genes in BCG that use episomal vectors are frequently unstable in the absence of the selection.[12-16] Integrative vectors, derived from temperate mycobacteriophages, such as L5[17] or Ms6,[16] have also been developed. These integration-proficient vectors encode integrase functions that allow a recombination event between the phage *attP* and the bacterial homologous *attB* sites. These vectors are more stable, but are integrated into the mycobacterial genome as a single copy.[17,18] The lack of stability of episomal vectors and the low copy number of integrative vector can compromise the

expression of heterologous antigen in BCG and influence the immune response to foreign antigens.

Expression systems currently used in BCG rely on antibiotic resistance genes as selectable markers.[11] However, these markers do not provide selection for the vaccine *in vivo*, nor are they desirable in a practical recombinant BCG based vaccine. Therefore, the development of more stable plasmid vectors for heterologous antigen expression in BCG that do not carry antibiotic resistance marker is necessary. Superinfection immunity to mycobacteriophages[19] and resistance to mercury[20] have been used as selectable markers in mycobacteria, but these still do not provide *in vivo* selective pressure.

In this report, we describe the construction of a BCG expression system using auxotrophic complementation as a selectable marker. A BCG Δ*leuD* was obtained by gene knock out, and a plasmid encoded *leuD* gene used as a selectable marker. The kanamycin resistance gene, necessary for selection in *E. coli* during plasmid construction, was removed prior to BCG transformation by two different strategies: bacteriophage P1 Cre-*loxP* site-specific recombination or restriction enzyme digestion followed by re-ligation. The new selection system resulted in remarkably improved stability of the vector as the selective pressure is maintained when the rBCG is inside macrophage cells.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Escherichia coli strain TOP10 (Invitrogen) was grown in Luria-Bertani medium at 37 °C with the addition of the appropriate antibiotic (kanamycin 50 µg/ml or hygromycin 200 µg/ml). *M. bovis* BCG Pasteur and BCG Δ*leuD* were grown in Middlebrook 7H9 broth (Difco) supplemented with 10% of oleic acid, albumin, dextrose complex (OADC – Difco), 0.2% glycerol and 0.05% Tween 80 (Sigma), or 7H10 and 7H11 agar (Difco) containing 10% OADC and 0.2% glycerol. *M. smegmatis* mc^2155 was grown in Middlebrook 7H9 broth (Difco) or Nutrient Broth II supplemented with 0.2% glycerol and 0.05 % Tween 80, or 7H10 agar (Difco) with 0.2% glycerol. When necessary, the auxotrophic strains

were grown in media supplemented with 100 μg/ml L-leucine (Sigma), 25 μg/ml kanamycin (Sigma) or 50 μg/ml hygromycin B (Invitrogen) and 40 μg/ml X-gal (Sigma).

2.2. Experimental animals

BALB/c mice (4-6 week old) were obtained from the Central Animal Facility of the Federal University of Pelotas. The experimental animals were housed at the animal facility of the Biotechnology Centre of the Federal University of Pelotas (UFPel). The animals were maintained in accordance with the guidelines of the UFPel Ethics Committee in Animal Experimentation throughout the experimental period.

2.3. Construction of *leuD* gene knock-outs in *M. bovis* BCG Pasteur strain 1173P2 and *M. smegmatis* mc^{2}155

Unmarked BCG Δ*leuD* and *M. smegmatis* Δ*leuD* were constructed as described by Parish and Stoker.[21] This strategy involves PCR amplifying chromosomal regions from either side of the gene of interest and cloning them into a suicide vector containing kanamycin resistance, *lacZ* and the counter selective *sacB* gene. When transformed into mycobacteria the plasmid integrates into the chromosome by a single cross-over to give $LacZ^+$, Kan^R colonies. Spontaneous double cross-overs, which result in wild type revertants or knock-outs, can then be selected for using *SacB* and screened.

The homologous chromosomal regions were PCR amplified from regions upstream (BCG-HR1-F 5' CTACCTGCAGCAACGTGCGGGCCTAACACACGGATA and BCG-HR1-R 5' CATCAAGCTTGGATCGAGGCCTTTGAGGGT) and downstream (BCG-HR2-F 5' CTCAAACGTTGCTGATCCCCTAGCTGTTCT and BCG-HR2-R 5' CATCGGATCCTAGCCACACCTCAACCCAC) of the BCG *leuD* and from the regions upstream (Ms-HR1-F 5' GCGACTGCAGATCGGAAAGGCAGCACCT and Ms-HR1-R 5' ATCCAAGCTTCCACCTAGTCGGTTCTGGAG) and downstream (Ms-HR2-F 5' ATCGAAGCTTGCTTCTCCTTCGTGGGTTTT and Ms-HR2-R 5' ATTAGGATCCCTCAAGCCCTTCAAGACCAT) of the *M. smegmatis leuD*. These were cloned into p2-NIL using *Pst*I/*Hin*dIII and

*Hin*dIII/*Bam*HI and the resultant plasmid ligated to the pGOAL-19 *Pac*I fragment. [22]

To transform the knock-out plasmids into mycobacteria, cultures in logarithmic growth at an OD_{600} of 0.6-1, were harvested by centrifugation. The pellet was washed in ×1, ×0.5 and ×0.2 volumes of 10% glycerol (37 °C for BCG and 4 °C for *M. smegmatis*) and the cells finally resuspended in ×0.01 volume. A 200 μl aliquot of competent cells were transformed by electroporation (25 μF, 1000 Ω, 2.5 V for BCG and 25 μF, 600 Ω, 2.5 V for *M. smegmatis*) with approximately 1 μg UV irradiated (100 mJ/cm^2) plasmid. BCG cells were recovered in 10 ml 7H9 for 12-24 h and plated on 7H11 with kanamycin and X-Gal while *M. smegmatis* cells were recovered for 4 h in Nutrient Broth II and plated on Nutrient Agar II with kanamycin and X-Gal. Colonies with β-galactosidase activity were sub-cultured onto plates containing X-Gal and 10% sucrose. White colonies were selected and their genotype determined by PCR using BCG-HR1-F and BCG-HR2-R, and Ms-HR1-F and Ms-HR2-R; and by Southern blot using probes containing the homologous regions, the *leuD* gene and pGOAL-19, The auxotrophy of the Δ*leuD* isolates of both BCG and *M. smegmatis* was tested by culturing in 7H9 and on 7H10 media, with and without leucine.

2.4. Auxotrophic complementation vector construction

A schematic representation of the steps involved in the design of the vectors is provided in Fig. 1. The *leuD* gene coding sequence was PCR amplified from *M. bovis* BCG P3 DNA with primers *leuD*I (5' AATCTAGAACAGCTAGGGGATC), and *leuD*II (5' TCCTGCAGTTCTACGCCTCA). The amplified fragment was digested with *Xba*I and *Pst*I and cloned into pUS977[13] to generate pUP400. The Hyg^R cassette from pGOAL19[22] was obtained by *Kpn*I digestion and ligated into *Kpn*I digested pUP400 to give pUP401.

Two methods were used to enable the kanamycin resistance gene to be removed from the vector prior to transformation into BCG. To remove the resistance gene by digestion and re-ligation, an adaptor that carries the *Hin*dIII site with *Pac*I compatible ends was ligated into the *Pac*I site of pUP401. Adap-F

(5' GATATCAAGCTTAAGACGCGTTAA) and Adap-R (5' TAACGCGTCTTAAGCTTGATATCTA) were hybridized by boiling for 1 min and incubated for 3 h at room temperature. Hybridised oligonucleotides (500 ng) were ligated to *Pac*I digested pUP401 (200 ng). The ligation mix was heated at 70 °C for 10 min, and immediately electrophoresed on 1% agarose gel. The ligation product was excised and eluted from the gel using GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Biosciencies). Hybridization buffer (10 mM Tris-HCl pH 8.5; 100 mM NaCl; 1 mM EDTA) was added to the eluted DNA and the mix heated at 80 °C for 5 min, kept at room temperature for 3 h and used to transform *E. coli* TOP10 cells. Recombinant clones were identified by digestion with *Hin*dIII and by DNA sequencing (MegaBACE - Amersham Biosciences). The resultant plasmid was named pUP402.

To enable the kanamycin resistance gene to be removed by site-specific recombination, oligonucleotides containing the cre-*loxP* site with either terminal *Hin*dIII sites or terminal *Pac*I sites were synthesized. The cre-*loxP*-*Hin*dIII F (5' TATAACTTCGTATAATGTATGCTATACGAAGTTAT) and cre-*loxP*-*Hin*dIII R (5' ATAACTTCGTATAGCATACATTATACGAAGTTATA) oligonucleotides were hybridized and ligated into *Hin*dIII digested pUP401 vector to give pUP401-lox-*Hin*dIII. Cre-*loxP* *Pac*I F (5' TAAATACTTCGTATAATGTATGCTATACGAAGTTATAT) and Cre-*loxP* *Pac*I R (5' ATATAACTTCGTATAGCATACATTATACGAAGTATTTA) oligonucleotides were hybridized and ligated into pUP401-lox-*Hin*dIII digested with *Pac*I as described for the *Hin*dIII-*Pac*I adaptor. The presence and orientation of the *loxP* sites were determined by restriction digestion and sequencing. The resulting vector was named pUP404.

To assess functional stability of the *leuD* complementation system *in vivo*, a *lac*Z gene in fusion with *hsp60* or 18kDa promoters was used. The *lac*Z-*hsp*60 and *lac*Z-18kDa cassettes were PCR amplified from pUS985 and pUS993 vectors respectively,[13] using Hsp60 F (5' TCGGTACCCCGACCACAACGACG), and 18kDa F (5' TCGGTACCGCAGCGACGGCACCGG) with the *lac*Z R primer (5'TCGGTACCTTATTTTTGACACCAGAC). The fragments were ligated into the *Kpn*I site of the pUP402 vector, replacing the hyg^{R} gene. The resulting vectors were named pUP402+*lacZ*/*hsp*60 and pUP402+*lacZ*/18kDa.

2.5. Removal of the kanamycin resistance gene

To remove the kanamycin resistance gene by digestion/re-ligation, pUP402 (1 μg) was digested with *Hin*dIII and re-ligated with T4 DNA ligase. To remove the kanamycin resistance gene by *in vitro* site-specific recombination, pUP404 (1 μg) was incubated for 1 h with 3 U Cre Recombinase (Biolabs). *M. bovis* BCG Δ*leu*D and *M. smegmatis* mc^{2}155 Δ*leuD* electrocompetent cells were transformed with the product of the ligation reaction (pUP402 Δkan^R) or recombination reaction (pUP404 Δkan^R). Transformants were plated on 7H10 with and without kanamycin and the colony counts compared to evaluate the efficiency of kanamycin resistance gene removal. These experiments were carried out in triplicate.

2.6. *In vitro* stability analysis

Cultures of *M. smegmatis* mc^{2}155 Δ*leuD* transformed with pUP402 Δkan^R vector; BCG Δ*leu*D and BCG Pasteur transformed with pUP402, pUP402 Δkan^R, pUP402+*lacZ/hsp*60 or pUP402+*lacZ*/18kDa were grown in 7H9 broth with and without selection (L-leucine for Δ*leu*D strains and kanamycin for rBCG Pasteur). Transformed BCG Δ*leuD* and *M. smegmatis* mc^{2}155 Δ*leuD* were subcultured seven times and twenty times respectively, by transferring 62.5 μl of the stationary phase culture to 2 ml of fresh medium every seven days for BCG and every two days for *M. smegmatis* mc^{2}155. Every three or four subcultures, the number of colonies on selective and non-selective 7H10 plates containing X-Gal were compared. Every four subcultures total DNA was extracted, transformed into *E. coli* TOP10 cells and plated on LB agar containing hygromycin for pUP402 Δkan^R, or kanamycin for pUP402+*lacZ*/*hsp*60, pUP402+*lacZ*/18kD and pUP402. The restriction digestion banding pattern of plasmids from five individual colonies from each transformation were determined in order to evaluate the plasmid's structural stability. These experiments were repeated three times.

2.7. β-galactosidase Assay

β-galactosidase activity was assayed in BCG Δ*leuD* and BCG Pasteur as previously described.[23] Briefly, duplicate 0.2 ml volume of test cultures (OD_{600}

0.8-1.4) were added to 0.3 ml Z buffer (60 mM $_2NaH_2PO_4$, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mecaptoethanol) and 0.5 ml of glass beads 0.1 mm (Biospec Products). The cells were lysed in a Hybaid Ribolyser (Thermo Hybaid) for 40 sec at power 5, centrifuged and the supernatant incubated at 28 °C for 5 min. A volume of 200 µl ONPG 4 mg/ml (Sigma) was added and the reaction incubated at 28 °C. When sufficient yellow colour developed, the reaction was stopped by adding 200 µl of 1 M Na_2CO_3 and the incubation time recorded. The OD_{420} of each reaction mix was measured and the β-galactosidase activity was calculated.[23]

2.8. *In vivo* stability analysis of the rBCG

Four to six week-old BALB/c mice were used to evaluate the *in vivo* stability of the rBCG. In the first experiment forty-eight animals were randomly allocated into six groups, eight animals per group, and inoculated intraperitoneally with approximately 5 x 10^5 cfu of rBCG in 100 µl of sterile PBS-T. Group one was inoculated with rBCG Δ*leuD* (pUP402+*lacZ/hsp*60), group two with rBCG Δ*leuD* (pUP402+*lacZ/18kDa*), group three with rBCG Pasteur (pUP402+*lacZ/hsp*60), group four with rBCG Pasteur (pUP402+*lacZ/18kDa*), group five with rBCG Δ*leuD* (pUP402), and group six with rBCG Pasteur (pUP402). At weeks 2, 5, 8, 12 , 16 and 30 after inoculation the spleen from one mouse per group was removed, homogenized, serially diluted in 7H9, and plated onto 7H10 with X-Gal and with or without selection (L-leucine for rBCG Δ*leu*D, and kanamycin for rBCG Pasteur). A second experiment was carried out with the same number of animals per group, however spleen from two mice was removed at each time point. The number of resultant colonies was compared and the percentage of blue colonies determined.

3. RESULTS

3.1. *M. bovis* BCG *ΔleuD* and *M. smegmatis ΔleuD* construction

Unmarked *M. bovis* BCG *ΔleuD* and *M. smegmatis ΔleuD* were successfully constructed by homologous recombination using the strategy of Parish and Stoker.[22] Genotypes were confirmed by PCR and Southern blots,

both giving bands that agreed with the predicted knock-out genotype. The resultant Δ*leuD* strains were not able to grow on 7H10 or in 7H9 without the addition of leucine.

3.2. Evaluation of *leu*D as a selectable marker

In order to evaluate the usefulness of auxotrophic complementation as a selectable marker, the *leuD* coding sequence was amplified by PCR and cloned into pUS977 under the control of P_{AN} promoter. The resulting vector, named pUP400 (Fig. 1), was used to transform BCG Δ*leuD* and *M. smegmatis* mc^{2}155 Δ*leuD*. Transformation rates were similar using either auxotrophic complementation or kanamycin resistance selection methods, approximately 6 x 10^2 cfu/μg DNA for *M. bovis* BCG Δ*leuD*, and 3.6 x 10^3 cfu/μg of DNA for *M. smegmatis* mc^{2}155 Δ*leuD*. Thus, the *leuD* complementation system and kanamycin resistance had equivalent selective capacities.

3.3. Removal of the kanamycin resistance gene

Two different approaches were evaluated to remove the kanamycin resistance gene from the plasmid prior to transformation into mycobacteria: restriction enzyme digestion followed by re-ligation of the vector; and bacteriophage P1 Cre-*loxP* site-specific recombination system. An oligonucleotide containing a *Hin*dIII site was cloned downstream of the kanamycin gene, resulting in two *Hin*dIII sites, one either side of the kanamycin gene. Digestion and re-ligation of the resulting vector, named pUP402, was carried out just before transformation of both BCG Δ*leuD* or *M. smegmatis* mc^{2}155 Δ*leuD.*

The second approach consisted of cloning oligonucleotides containing Cre-*loxP* sites flanking the kanamycin resistance gene. *In vitro* treatment of pUP404 with Cre Recombinase resulted in excision of the kanamycin resistance gene. The DNA band corresponding to the kanamycin resistance gene excised from the plasmid after treatment with Cre Recombinase could be observed by agarose gel electrophoresis (data not shown).

Transformation efficiency with re-ligated DNA or with Cre Recombinase treated DNA was approximately one log lower than with intact plasmid. The

efficiency of removal of the kanamycin gene was evaluated by replica plating one hundred individual transformants on plates with and without kanamycin. For pUP402 digested with *Hin*dIII and re-ligated, 95% of the colonies failed to grow on kanamycin, confirming that the kanamycin resistance gene had been efficiently removed. The same vector when used to transform *M. smegmatis* mc^2155 Δ*leuD* revealed a removal efficiency of 85%. Removal of the kanamycin resistance gene from pUP404 by site-specific recombination was less efficient. Only 41% of *M. bovis* BCG Δ*leuD* transformed with Cre Recombinase treated vector lost the kanamycin resistance gene, whereas in *M. smegmatis* mc^2155 Δ*leuD* this number was 31%.

3.4. *In vitro* stability analysis of recombinant mycobacteria

To evaluate the *in vitro* persistence of the plasmids harbouring the auxotrophic complementation *leu*D gene, subcultures on media with and without selection were carried out. All of the *M. smegmatis* mc^2155 Δ*leuD* strain cultured on selective medium (without leucine supplementation) maintained the vector for over 20 subcultures (approximately 190 generations). However, only 20% of the colonies still carried the vector after only four subculturing passages (approximately 40 generations) without selective pressure (with leucine). This decreased to only 4% after 20 passages (Fig. 2).

In order to evaluate the functional stability of genes carried by the complementation system, *lacZ* was used as a reporter gene under two different mycobacterial promoters: the *hsp60* promoter (pUP402+*lacZ/hsp60*) and the 18kDa promoter from *M. leprae* (pUP402+*lacZ/18kDa)*. Both vectors were used to transform *M. bovis* BCG Δ*leuD* and *M. bovis* BCG Pasteur. When bacteria were grown under selective pressure, the level of expression of *lacZ* determined by β-galactosidase enzyme activity assay, remained stable with successive passages, however in the presence of leucine for rBCG Δ*leuD* or absence of kanamycin for rBCG Pasteur, the level of expression dropped. In the case of BCG Pasteur transformed with pUP402+*lacZ/hsp60* and grown under non-selective condition, after 6 passages β-galactosidase activity was completely abolished. To ascertain that this effect was not due to mutations in the expression cassette, cultures grown without selective pressure were plated out

on selective and non-selective media containing X-Gal. The small number of colonies obtained on selective medium was blue, whereas on non-selective medium a large number of white colonies were obtained, demonstrating that the drop in *LacZ* expression was as a result of loss of the vector.

Structural stability of the vectors was evaluated by plasmid rescuing and analysis of restriction digest patterns. Every four subcultures of *M. smegmatis* mc^2155 Δ*leuD* transformed with pUP202 Δkan^R or rBCG Δ*leuD* (pUP402+*lacZ*/*hsp*60, pUP402+*lacZ*/*18kDa* and pUP402) and rBCG Pasteur (pUP402+*lacZ*/*hsp*60, pUP402+*lacZ*/*18kDa* and pUP402) total DNA was extracted and used to transform *E. coli*. Hygromycin selection was used throughout the plasmid rescuing experiments to distinguish between un-transformed, plasmid-free bacteria as some vectors had the kanamycin resistance gene removed. Plasmid DNA was extracted from individual colonies and restriction digested. The resulting restriction patterns were compared with those of the vectors originally used to transform the mycobacterial hosts. Gross modifications in vector structure were not observed (data not shown).

3.5. *In vivo* stability analysis of the rBCG

Stability of *M. bovis* BCG Δ*leu*D and *M. bovis* BCG Pasteur transformed with plasmids containing the *leuD* gene as selectable marker was evaluated in mice. Three different plasmids were used to transform both BCG strains. Initial selection was carried out on medium without leucine for BCG Δ*leu*D or containing kanamycin for BCG Pasteur. Approximately 5 x 10^5 cfu of each strain were inoculated into mice. Auxotrophic complementation vectors used to transform BCG Δ*leu*D showed 90-100% of stability *in vivo* during the 30 weeks of the experiment (Fig. 3). There was no significant difference among vectors in terms of stability. Conversely, the same vectors used to transform BCG Pasteur were lost during *in vivo* growth. Five weeks post inoculation less than 20% of the bacterial cells still had the plasmid. This number fell to zero at 16 weeks post inoculation (Fig. 3). A very high level of functional stability was also observed as all the recovered BCG Δ*leu*D transformed with vectors carrying *lacZ* gave rise to blue colonies when plated onto medium containing X-Gal. Interestingly, it was noted that there was no significant reduction in the number

of cfu recovered from mice inoculated with BCG Δ*leuD* complemented strains in comparison to rBCG Pasteur, showing that auxotroph complementation does not come at the cost of decreased rBCG survival.

4. DISCUSSION

A highly stable plasmid vector for cloning and expression of foreign genes in BCG has been developed in this study. This system is based on the use of a *M. bovis* BCG strain auxotrophic for the leucine amino acid obtained by knocking out the *leu*D gene, and complementation with *leu*D inserted into the plasmid vector, which acts as a selectable marker. Auxotrophic BCG strains, when inside macrophages, are unable to access intracellular amino acids, thus they fail to grow *in vivo*.[24] This important observation opened the possibility of using auxotrophic complementation as a selectable marker, an approach that has two main advantages: it provides active selection *in vivo*, unlike antibiotic resistance markers, and it abolishes the need for using an antibiotic resistance gene as a vector component.

The majority of currently used mycobacterial vectors carry the *Tn*5 or *Tn*903-derived kanamycin resistance gene that codes for the enzyme aminoglycoside phosphotransferase.[11,18] This enzyme inactivates the antibiotic kanamycin and permits bacterial cells to grow in the presence of the drug. Mercury resistance and superinfection immunity to mycobacteriophage L5 have also been used as selectable markers in mycobacteria, but with limited success.[19,20,25] None of the selectable markers used so far are active *in vivo*. For many applications this is not an issue, but for expression of foreign antigens with the aim of developing BCG into a multivalent recombinant vaccine, or an improved vaccine against tuberculosis, *in vivo* vector stability is critical. Although several studies have demonstrated that even without *in vivo* selective pressure the vector can persist for a long time, loss of the plasmid vector has been implicated in failure of recombinant BCG vaccine candidates.[16,26]

Auxotrophic complementation has been used as selectable marker for stabilizing the plasmid vector during fermentation in *E. coli*,[27] for yeast transformation[28,29] and for replacing antibiotic resistance marker in

Pseudomonas fluorescences fermentation.[30] However, to the best of our knowledge, this is the first report of the use of auxotrophic complementation as a selectable marker in mycobacteria. The use of antibiotic-resistance genes as selectable markers in genetically modified organisms (GMO) is coming under increased scrutiny, for fear that they may spread to human pathogens, thereby reducing the effectiveness of antibiotic therapy. This is of particular concern when the GMO is a live bacterial vaccine, therefore a recombinant BCG vaccine carrying an antibiotic resistance gene is unlikely to be accepted.

The new selection system described here not only is highly stable *in vivo*, as the selective pressure for the vector is maintained, but also eliminates the problem of having an antibiotic resistance gene as part of the vector. However, to allow the selection and maintenance of the plasmid in *E. coli* during construction kanamycin resistance gene or other antibiotic resistance marker is still necessary. The possibility of using an *E. coli* Δ*leuD* host to construct the vector was considered, however, the identity between mycobacterial and *E. coli* *leuD* is low, therefore it is unlikely that the mycobacterial *leuD* gene would be able to complement an auxotrophic *E. coli*. Initial attempts to complement BCG Δ*leuD* with the *E. coli* *leu*D were not successful, ruling out its use as a selectable marker. As a result, the antibiotic resistance marker has to be removed just before transferring the vector to BCG.

With the aim of developing an efficient mechanism of removal of the kanamycin resistance gene, the Cre-*loxP* site specific recombination system[31] was employed. Two lox sites were cloned flanking the kanamycin resistance gene. Incubation of the plasmid vector with Cre recombinase resulted in deletion of the fragment located between the lox sites with a relatively low efficiency. A simpler approach was also developed by inserting an oligonucleotide containing a *Hin*dIII site downstream of the kanamycin resistance gene, resulting in two *Hin*dIII sites flanking the gene. Digestion with *Hin*dIII followed by re-ligation using a concentration of DNA that favoured intra molecular ligation resulted in removal of the kanamycin gene with a significantly higher efficiency than that obtained with the Cre-*loxP* system. In the end, both systems were functional and either could be used. The Cre-*loxP* system must be used when the foreign gene has an internal *Hin*dIII site. In other situations

the vector with the two *Hin*dIII sites flanking the kanamycin resistance gene will be the best choice.

The use of auxotrophic complementation as selectable marker for expression of foreign genes in BCG described in this report provides stability previously described only for integrative vectors,[16,32] with the advantages of a multicopy vector, namely higher levels of foreign antigen expression. In addition, since no antibiotic resistance gene is required, the system is applicable to the development of recombinant live vaccines. Evaluation of the immune response obtained with the use of this new selection system is currently been performed with several recombinant antigens. It is expected that it will be able to elicit a strong and long-lasting immune response against these antigens, contributing to the development of BCG into a multivalent vaccine.

5. ACKNOWLEDGMENTS

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6. REFERENCES

1. Stover CK, Delacruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, et al. New use of BCG for recombinant vaccines. *Nature* 1991;**351**:456-60.

2. Bloom BR, Fine PEM. The BCG experience: implications for future vaccines against tuberculosis. In: Bloom BR, ed. *Tuberculosis: pathogenicity, protection and control.* Washington DC: American Society for Microbiology, 1994:p. 531-57.

3. Abdelhak S, Louzir H, Timm J, Blel L, Benlasfar Z, Lagranderie M, et al. Recombinant BCG expressing the *Leishmania* surface-antigen Gp63 induces protective immunity against *Leishmania major* infection in BALB/c mice. *Microbiology* 1995;**141**:1585-92.

4. Bastos RG, Dellagostin OA, Barletta RG, Doster AR, Nelson E, Zuckermann F, et al. Immune response of pigs inoculated with *Mycobacterium bovis* BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. *Vaccine* 2004;**22**:467-74.

5. Matsumoto S, Yukitake H, Kanbara H, Yamada T. 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* 1998;**188**:845-54.

6. Ohara N, Yamada T. Recombinant BCG vaccines. *Vaccine* 2001;**19**:4089-98.

7. Horwitz MA. Recombinant BCG expressing *Mycobacterium tuberculosis* major extracellular proteins. *Microbes Infect* 2005;**7**:947-54.

8. Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. 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 USA* 2000;**97**:13853-8.

9. Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. A novel live recombinant mycobacterial vaccine against bovine tuberculosis more potent than BCG. *Vaccine* 2006;**24**:1593-600.

10. Labidi A, David HL, Roullanddussoix D. Cloning and expression of mycobacterial plasmid DNA in *Escherichia coli*. *FEMS Microbiol Lett* 1985;**30**:221-5.

11. Snapper SB, Lugosi L, Jekkel A, Melton RE, Kieser T, Bloom BR, et al. Lysogeny and transformation in mycobacteria - stable expression of foreign genes. *Proc Natl Acad Sci USA* 1988;**85**:6987-91.

12. Lagranderie M, Murray A, Gicquel B, Leclerc C, Gheorghiu M. Oral immunization with recombinant BCG induces cellular and humoral immune-responses against the foreign antigen. *Vaccine* 1993;**11**:1283-90.

13. Medeiros MA, Dellagostin OA, Armoa GRG, Degrave WM, 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.

14. Murray A, Winter N, Lagranderie M, Hill DF, Rauzier J, Timm J, et al. 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* 1992;**6**:3331-42.

15. Nascimento IP, Dias WO, Mazzantini RP, Miyaji EN, Gamberini M, Quintilio W, et al. Recombinant *Mycobacterium bovis* BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live *Bordetella pertussis* in mice. *Infect Immun* 2000;**68**:4877-83.

16. Mederle I, Bourguin I, Ensergueix D, Badell E, Moniz-Peireira J, Gicquel B, et al. Plasmidic versus insertional cloning of heterologous genes in *Mycobacterium bovis* BCG: impact on in vivo antigen persistence and immune responses. *Infect Immun* 2002;**70**:303-14.

17. Lee MH, Pascopella L, Jacobs WR, Hatfull GF. Site-specific integration of mycobacteriophage-L5 -integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and Bacille Calmette-Guerin. *Proc Natl Acad Sci USA* 1991;**88**:3111-5.

18. Dellagostin OA, Wall S, Norman E, Oshaughnessy T, Dale JW, McFadden J. Construction and use of integrative vectors to express foreign genes in mycobacteria. *Mol Microbiol* 1993;**10**:983-93.

19. Donnellywu MK, Jacobs WR, Hatfull GF. Superinfection immunity of mycobacteriophage-L5 - applications for genetic transformation of mycobacteria. *Mol Microbiol* 1993;**7**:407-17.

20. Baulard A, Escuyer V, Haddad N, Kremer L, Locht C, Berche P. Mercury resistance as a selective marker for recombinant mycobacteria. *Microbiology* 1995;**141**:1045-50.

21. Parish T, Stover NG. Mycobacteria protocols. New Jersey: Humana Press Inc., 1998.

22. Parish T, Stoker NG. Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis tlyA plcABC* mutant by gene replacement. Microbiology 2000;**146**:1969-75.

23. Dellagostin OA, Esposito G, Eales LJ, Dale JW, McFadden J. Activity of mycobacterial promoters during intracellular and extracellular growth. *Microbiology* 1995;**141**:1785-92.

24. Bange FC, Brown AM, Jacobs WR. Leucine auxotrophy restricts growth of *Mycobacterium bovis* BCG in macrophages. *Infect Immun* 1996;**64**:1794-9.

25. Reyrat JM, Pelicic V, Gicquel B, Rappuoli R. Counterselectable markers: Untapped tools for bacterial genetics and pathogenesis. *Infect Immun* 1998;**66**:4011-7.

26. 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.

27. Fiedler M, Skerra A. proBA complementation of an auxotrophic *E. coli* strain improves plasmid stability and expression yield during fermenter production of a recombinant antibody fragment. *Gene* 2001;**274**:111-8.

28. Takahashi T, Nozawa M, Hara S, Mizoguchi H. High fidelity segregation of a YEp vector in [cir0] strains of the yeast *Saccharomyces cerevisiae*. *J Biosci Bioeng* 2003;**96**:413-20.

29. Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev* 2000;**24**:45-66.

30. Schneider JC, Jenings AF, Mun DM, McGovern PM, Chew LC. Auxotrophic markers *pyrF* and *proC* can replace antibiotic markers on protein production plasmids in high-cell-density *Pseudomonas fluorescens* fermentation. *Biotechnol Prog* 2005;**21**:343-8.

31. Sternberg N, Hamilton D. Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol* 1981;**150**:467-86.

32. Vultos TD, Mederle I, Abadie V, Pimentel M, Moniz-Pereira J, Gicquel B, et al. Modification of the mycobacteriophage Ms6 attP core allows the integration of multiple vectors into different tRNAala T-loops in slow- and fast-growing mycobacteria. *BMC Mol Biol* 2006;**7**:47.

FIGURE CAPTIONS

Figure 1: Schematic representation of the strategy employed in this study for the construction of pUP vector series. The *lacZ* gene replaced the hygromycin resistance gene in pUP402+*lacZ*/*18kDa* and pUP402+*lacZ*/*hsp*60.

Figure 2: *In vitro* stability of pUP401 in *M. smegmatis* mc^2155 Δ*leuD* grown for successive passages on selective (no leucine) or non-selective (with the addition of leucine) media. The percentage represents the number of CFU that still maintained the vector (growth on selective medium) in relation to the total number of CFU. Twenty subcultures represent approximately 190 generations.

Figure 3. *In vivo* stability of rBCG transformed with vectors containing auxotrophic complementation. Mice were inoculated with BCG Δ*leu*D transformed with pUP402, pUP402+*lacZ/hsp*60 or pUP402+*lacZ*/18kD and BCG Pasteur transformed with the same vectors. Bacteria were recovered from spleen of three animals killed at 2, 5, 8, 12, 16 and 30 weeks post inoculation and plated on selective and non-selective medium. Ratios of resistant (rBCG) versus total BCG colonies were calculated for each strain. Average of the three BCG Δ*leuD* and three BCG Pasteur strains is shown.

Figure 1. Borsuk et al.

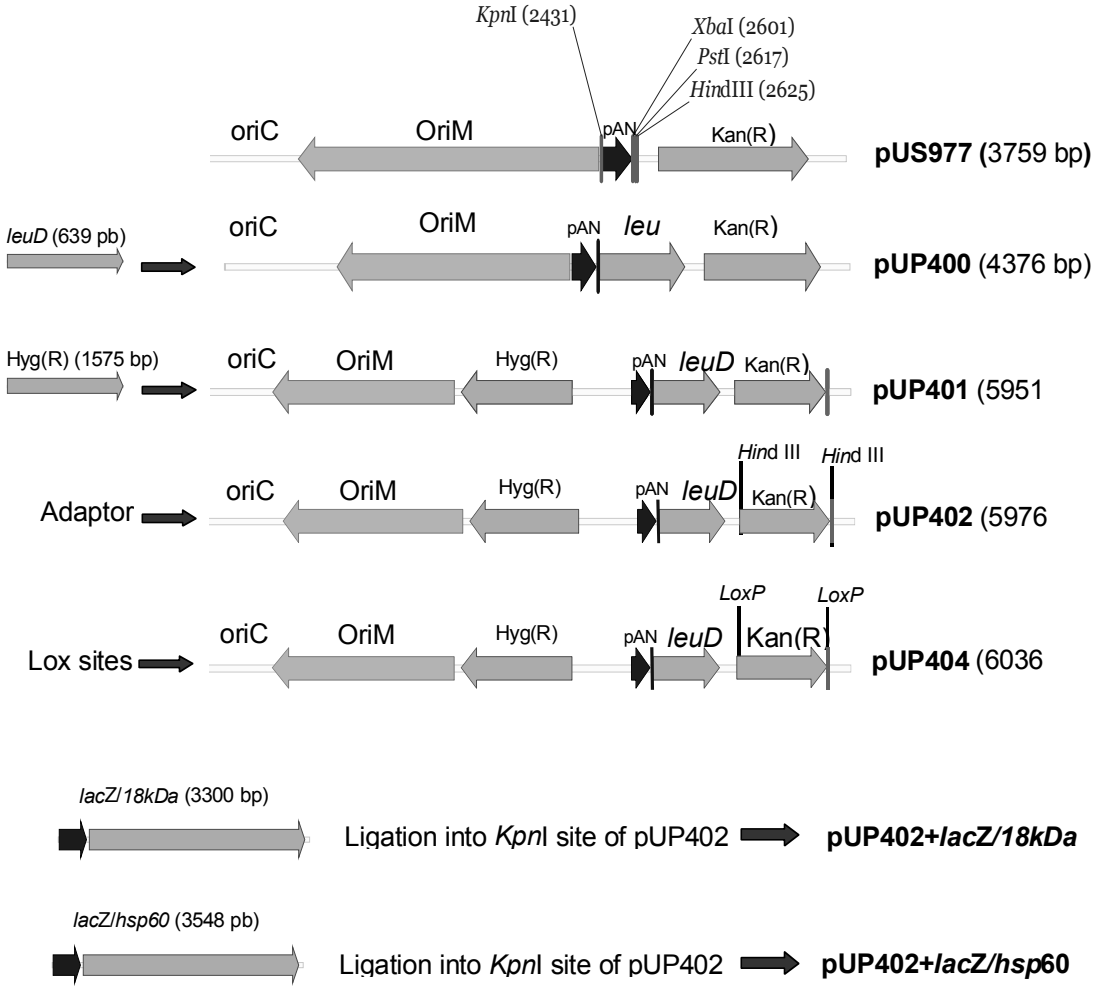


Figure 2. Borsuk et al.

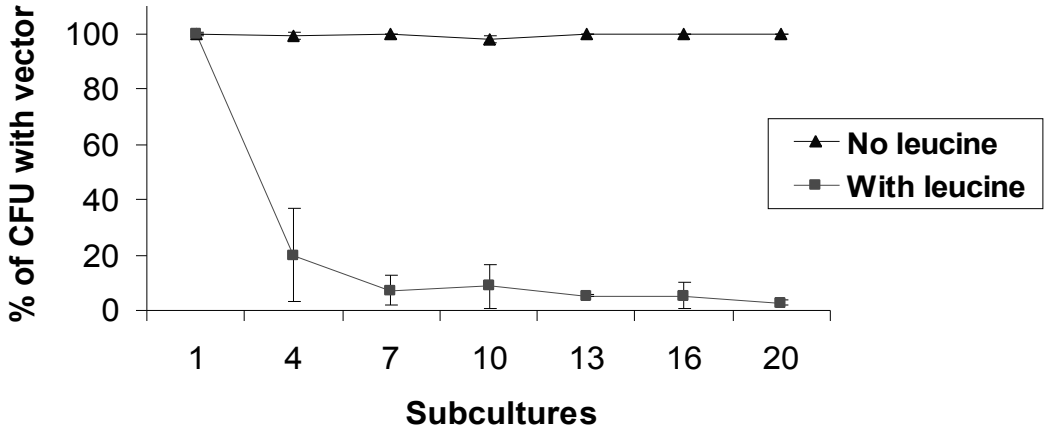
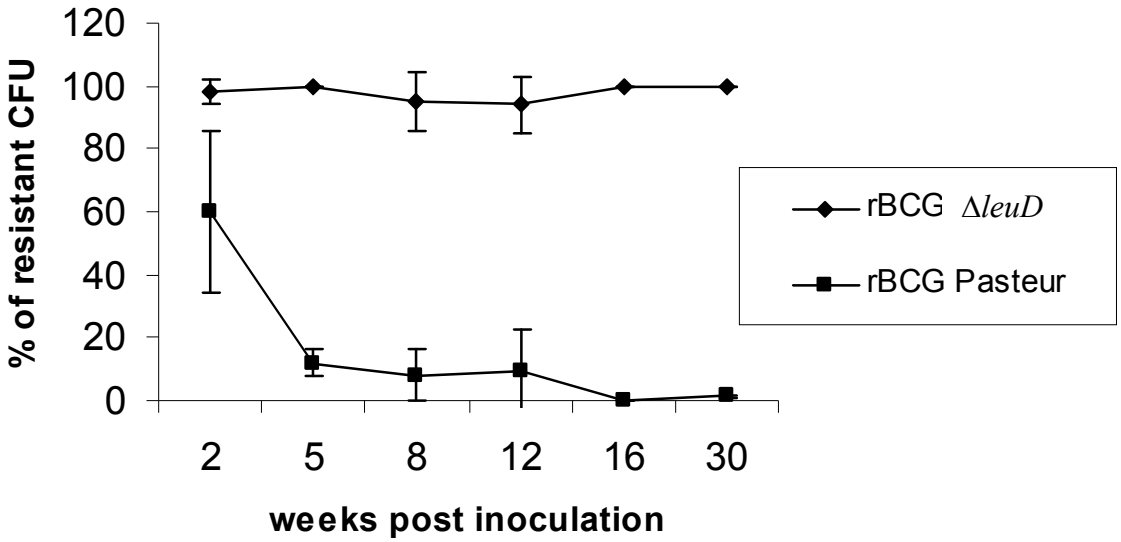


Figure 3. Borsuk et al.



4 ARTIGO 3

IDENTIFICATION OF PROTEINS FROM TUBERCULIN PURIFIED PROTEIN DERIVATIVE (PPD) BY LC-MS/MS

(Artigo e ser submetido ao periódico *Tuberculosis*)

IDENTIFICATION OF PROTEINS FROM TUBERCULIN PURIFIED PROTEIN DERIVATIVE (PPD) BY LC-MS/MS

Sibele Borsuk[a]; Jane Newcombe[b], Tom Mendum[b], Odir Antonio Dellagostin[a]
Johnjoe McFadden[b]

[a] *Centro de Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil,*
[b] *School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, UK.*

[*] Corresponding author
Centro de Biotecnologia, Universidade Federal de Pelotas. CP 354, 96010-900, Pelotas, RS, Brazil, Phone: +55 53 32757350. E-mail: odirad@terra.com.br.

ABSTRACT

The tuberculin purified protein derivative (PPD) is a widely used diagnostic antigen for tuberculosis, however it is poorly defined. Most mycobacterial proteins are extensively denatured by the procedure employed in its preparation, which explains previous extensive difficulties in isolating defined constituents from PPD to characterize their behaviour in B- and T-cell reactions. We characterized PPD components by LC-MS/MS, which combines the solute separation power of HPLC, with the detection power of a mass spectrometer. A total of 147 proteins among the five PPD samples (2 bovine PPD and 3 avium PPD) were identified. The majority of the proteins was cytoplasmic (81%) and involved in intermediary metabolism and respiration (29.9%). Bovine PPD had a considerable higher number of proteins comparing to avium PPD. We identified a group of 28 proteins that are present in both bovine PPD but absent in avium PPD preparations. In addition, five proteins found in bovine PPD are deleted in *M. bovis* BCG vaccinal strain. This study provides a comprehensive picture of PPD components leading to the identification of additional antigens useful as reagents for specific diagnosis of tuberculosis.

Key words: PPD, LC-MS/MS, proteomic analysis.

1. INTRODUCTION

Tuberculosis continues to be a worldwide problem for both humans and animals [1]. Human tuberculosis is predominantly caused by *Mycobacterium tuberculosis*. Bovine tuberculosis, caused mainly by *M. bovis* is an important cause of economic losses and can be a zoonotic infection [2]. An important control strategy for the prevention of these diseases is the use of effective vaccines. The *M. bovis* bacillus Calmette-Guerin (BCG) vaccine, an attenuated strain of *M. bovis*, has been widely used for control of human tuberculosis despite controversy over its protective efficacy [3]. In cattle, BCG has been used in a series of trials with various degrees of protection against *M. bovis* challenge [4-6]. However, a major constraint in the use of attenuated mycobacterial vaccines such as BCG is that vaccination of humans or cattle interferes with detection of tuberculosis by tuberculin skin test. The development of tests, which can distinguish between infection with *M. tuberculosis* or *M. bovis* and vaccination, could greatly assist in the diagnosis of early infection as well as to enhance the use of tuberculosis vaccines on a wider scale.

The tuberculin skin test cannot distinguish between a *M. tuberculosis* infection and BCG vaccination or exposure to environmental mycobacteria. These cross-reactions are generally attributed to the presence in PPD of antigens shared by other *Mycobacterium* species [7,8]. PPD is prepared by heat sterilization of 6-week-old *M. tuberculosis, M. bovis or M. avium* grown in Sauton medium, followed by filter sterilization and protein concentration using ultrafiltration or ammonium sulfate precipitation [9,10]. Considering the widespread use of this immunological reagent, it is surprising that little is known about the active components of PPD [10-12]. Some proteins that are probably present in PPD preparations have been tested as diagnostic reagents. The recombinant proteins MPB59, MPB64, MPB70 and ESAT 6 were evaluated in a differential diagnostic test, but only ESAT 6 showed to be suitable for differentiating BCG-vaccinated animals from those infected with bovine tuberculosis [13,14]. Synthetic peptides derived from ESAT6, CFP-10, MPB64, MPB70 and MPB83 were tested alone or in combination, with limited success [15,16].

Mass Spectrometry (MS) represents an efficient tool to perform analysis due to its high mass accuracy, sensitivity, and ability to deal with complex mixtures and it has proven to be a method of choice for characterizing proteins samples. Moreover, elucidation of the entire genome sequence makes it possible to analyze the proteome of *M. tuberculosis* and *M. bovis.*

In this work we used the LC-MS/MS (Liquid Chromatography/Mass Spectrometry/Mass Spectrometry), which combines the solute separation power of HPLC, with the detection power of a mass spectrometer, to characterize the proteins that are present in bovine and avium PPD preparations. Characterization of proteins from PPD preparations will be useful to identify additional antigens to be used in more specific and sensitive tests that could even be able to differentiate BCG vaccinated from infected individuals.

2. MATERIAL AND METHODS

2.1. PPD sample

Avian PPD samples were obtained from the Instituto Biológico, São Paulo, Brazil, from the Instituto Tecnológico do Paraná, Curitiba, Brazil and from the Veterinary Laboratories Agency, Weybridge, Surrey, UK. Bovine PPD samples were obtained from the Instituto Tecnológico do Paraná and from the Veterinary Laboratories Agency.

2.2 .1-D gel electrophoresis

Fifty micrograms of PPD preparation (avium or bovine) were mixed with 25 µL of SDS loading buffer and boiled for 5 min prior to separation on a 10 cm long, 1 mm thick 18% SDS-polyacrylamide gel. The protein migration was allowed to proceed until the blue dye had migrated to the bottom of the gel. Protein bands were visualized by silver staining.

2.3. Sample digestion

Sliced gel bands were washed twice with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for 15 min at room temperature (RT). The gel pieces were dehydrated by incubating them with 50 mL 100% ACN for 20 min at RT. Proteins were reduced using 10 mM DTT and alkylated with 55 mM iodoacetamide (IAA); both in 25 mM NH_4HCO_3. The gel pieces were dehydrated with ACN as described above, and rehydrated in 25 mM NH_4HCO_3 containing 0.01 mg/mL modified trypsin (Promega). Proteins were digested by trypsin for 16–20 h at 37 °C. Then, the tryptic peptides were eluted by incubating the gel pieces with 50 mL 1% trifluoroacetic acid (TFA) for 20 min at RT. The supernatant containing tryptic peptides was collected. Additional peptides were extracted from gel pieces by incubation with 50 µL 0.1% TFA in 50% ACN for 20 min at RT, the supernatant was collected and added to the previous one. Finally, the extracted digested peptides were concentrated to 10 µL using Speed-Vac® (Eppendorf).

2.4. LC-MS/MS

Online LC-MS/MS was performed using the Ultimate HPLC equipment (LC packings) and the ESI-Q-TOF Ultima Global instrument (Waters, Micromass, Manchester, UK). Two different columns were used, either a capillary 0.3x150mm C18 RP column or a nano 0.075x150mm C18 RP in-house packed column. The flow rate was 3 µL/min for the capillary column and 0.2 µL/min for the nano column. The solvent gradient went from 5% B to 60% B in 42 min, then from 60% B to 95% B in 10 min, ending with constant 95% B for 5 min. Solvent A was aqueous 2% ACN in 0.1% TFA, whereas solvent B was aqueous 90% ACN in 0.1% TFA. Proteolytic peptide profiles were acquired in data-dependent MS/MS mode from 3 to 60 min with a maximum of 40 s spectral accumulation time, 10 s for each of a maximum of four selected peptides from each scan window. The electrospray voltage was set to 3 and 2.4 kV, respectively, for the capillary and nano setup. The collision energy was set to variable using charge recognition option.

2.5. Database Analysis

The obtained mass spectra were searched against the *M. tuberculosis* complex database using MASCOT Deamon software (v2.1.0, Matrix Science, London, UK). The search parameters were: 50 ppm tolerance as the minimum mass error, monoisotopic mass value and fixed modification of cysteine by carboxymethyl and variable modifications of oxidation and phosphorilation. A protein was regarded identified if the matched peptide mass fingerprint covered 20% of the complete protein sequence. An assignment with sequence coverage below 20% was only accepted if one or more of the main peaks were identified by MALDI MS/MS with a significant MASCOT score (above 95% certainty). The websites http://csc-fserve.hh.med.ic.ac.uk/msdb.html and http://genolist.pasteur.fr/TubercuList/index.html were used to identify the Rv numbers. To determinate the protein class, (C) cytoplasmic protein; (TMHMM) membrane protein; (TATP) Tat signal peptide; (SIGNALP) Sec signal peptide; (SECRETOMEP) Non-classical secretion, the tools available at http://www.cbs.dtu.dk/ and http://tbsp.phri.org websites were used.

3. RESULTS

3.1. Identification of proteins in PPD samples using LC-MS/MS

Five different PPD preparations, three avium PPD samples (two from Brazil - PPDavi BR1 and PPDavi BR2 and one from UK - PPDavi UK) and two bovine PPD, produced in Brazil (PPDbov BR) and in UK (PPDbov UK) were used in this study. A total of 147 proteins among the five PPD samples was identified by LC-MS/MS analysis based on at least one identified peptide per protein with a MASCOT confidence level above 95% (table 1). The identified proteins were classified according to the *M. tuberculosis* annotation. The number of proteins identified in the avium PPD preparations was lower comparing to the bovine PPD preparations. The PPDavi BR1 had 26 and the PPDavi BR2 had 28 different proteins identified by LC-MS/MS; the PPDavi UK also had 28 proteins identified. The proteins Rv0129c, Rv0216, Rv0350, Rv0440, Rv0652, Rv0685, Rv1827, Rv2244, Rv3418c, and Rv3663c were identified in all avium PPD samples. The proteins Rv0053, Rv0281, Rv0404,

Rv0828c, Rv1436, Rv1448c, Rv1475c, Rv1596, RV1687c, Rv1702c, Rv1837c, Rv2428, Rv2467, Rv2704, Rv2837c, Rv2943, Rv3028c, Rv3248c, and Rv3804c were identified only in the Brazilian avium PPD samples. Eight proteins (Rv1038c, Rv1608c, Rv1636, Rv1796, Rv2882, Rv2919c, Rv3244c, and Rv3874) were unique to the avium PPD from UK (table 1).

A higher number of proteins was identified in bovine PPD samples. PPDbov BR and PPDbov UK had, respectively, 61 and 105 different proteins identified (table 1). Nine proteins (Rv0216, Rv0350, Rv0440, Rv0652, Rv0685, Rv1827, Rv2244, Rv3418c and Rv3663c) were detected in all PPD preparations (avium and bovine). Of these, only one Rv0685, is a secreted protein. The others are cytoplasmic proteins. Forty six proteins were identified in both bovine preparations. Fifteen were unique to PPDbov BR and 59 to PPDbov UK. A group of 28 proteins (Rv0242c, Rv0248c, Rv0270, Rv0384c, Rv0467, Rv0623c, Rv0831c, Rv0896, Rv1093, Rv1133c, RV1630, Rv1745c, Rv1855c, Rv1916, Rv1926c, Rv1980c, Rv2593c, Rv2626c, Rv2873, Rv3001c, Rv3045, Rv3117, Rv3417c, RV3458c, Rv3716c, Rv3841, Rv3846, Rv3874) was identified in both bovine PPD preparations, but not in avium preparations (table 1).

The nucleotide sequence of five proteins identified in PPD preparations (Rv1980c, Rv1984c, Rv2346c, Rv3874, Rv3875) are delete in *M. bovis* BCG vaccinal strain. However, the Rv2346c and Rv3874 were also identified in avium PPD samples (table 1). The major secreted proteins like antigen 85 components, MPT32, MPT64, MPT83, MPT53 and MPT70 were present in PPD preparations.

3.2. Determination of protein location

Proteins were analyzed regarding their location on the cell. The predominant class was cytoplasmic proteins, accounting for 81.6%. Membrane or secreted proteins with different signal peptides (Tat, Sec and Non-Classical secretion) accounted for only 18.3% of the proteins present (table 1).

The most common protein class among the avium PPD was also cytoplasmic proteins. Only five proteins were classified as secreted. Of this,

Rv1860, Rv2875 and Rv3804c are also found in bovine preparations. Only Rv3244c was specific from avium preparation (PPDavi UK).

3.3. Functional distribution of the identified PPD proteins

The annotated *M. tuberculosis* H37Rv proteins have been classified into 12 distinct groups (http://genolist.pasteur.fr/TubercuList/index.html). The 147 proteins identified by LC-MS/MS in this study were distributed across ten functional groups (table 1). Most of the proteins identified are involved in intermediary metabolism and respiration (functional group 7, 26%), and prokaryotic cell wall and cell processes (functional group 3, 17.7%). In the avium PPD samples, the most common functional group was also intermediary metabolism and respiration, except in the PPDavi UK, in which the majority of the proteins (eight) belongs to functional group 3.

3.4. Molecular mass distribution of the identified PPD proteins

The protein with the lowest molecular mass in this study was 6.31 kDa (Rv2346c, Putative ESAT-6 like protein 6). The probable aconitate hydratase (Rv1745c), with a molecular mass of 102.7 kDa observed by LC-MS/MS represented the largest identified protein. The majority of the proteins were found in the range between 10 and 50 kDa (figure 1), the largest cluster was of proteins with 10-20 kDa.

3.5. Major PPD components

The major components in the PPD preparations were determined by the number of peptides identified by LC-MS/MS for each protein. The most frequent protein in all samples, except in PPDbov UK, was Rv3663c, a probable ABC transporter. In the bovine PPD from UK the protein EsxB (ESAT-6-like protein esxB, Rv3874), and Mpt70 protein (Rv2875) were the two most frequent proteins. Interestingly, the first one is not in the list of the 10 most frequent in the Brazilian PPD. The protein Mpt83 (Rv2873) was also among the most frequent in UK PPD, but not in Brazilian PPD (table 2). The protein groEL2 (60 kD chaperonin 2, Rv0440) was present in large amounts in all PPD samples.

The DnaK (70 kD heat shock protein, Rv0350) and protein EF-Tu (elongation factor EF-Tu, Rv0685) are also found in large amounts in all samples (table 2).

4. DISCUSSION

PPD is an immunological reagent widely used for diagnosis of tuberculosis, but knowledge about the components of this heat-inactivated culture filtrate of mycobacteria is very limited [10-12,17-19]. In this study a proteomic analysis of tuberculin purified protein derivative (PPD) samples used for diagnosis of animal tuberculosis was carried out in order to identify the proteins present in this immunological reagent.

The fact that current tuberculosis control in cattle is entirely based on the tuberculin skin test to identify infected animals, and the subsequent slaughter of such tuberculin-positive animals, limits the use of BCG vaccination, an important measure that could aid in the control bovine tuberculosis [20]. Although whole-blood gamma interferon assays have been developed for diagnostic of bovine tuberculosis [21,21,22], and depending on the antigens used, these tests can effectively differentiate vaccinated from infected animals [13,14,16], they are more expensive and difficult to implement in remote places. It would be desirable to have an alternative test with similar features to the current tuberculin test, that is, easy to perform under field conditions, inexpensive and that does not require sophisticated equipments and laboratory infrastructure.

The identification of proteins present in PPD preparations could allow the development of defined set of antigens to be used as a synthetic PPD. This set of antigens would contain those that are present in *M. bovis*, but absent in environment mycobacteria, increasing the specificity of the test. In addition it should be composed preferentially by antigens that are absent in *M. bovis* BCG, allowing differentiation between infected and BCG vaccinated animals. Such a test would allow the use of BCG vaccine in cattle and other domestic animals, a practice that is currently not allowed in most countries.

The number of proteins identified in the avium PPD preparations teste in our study was lower comparing with bovine PPD preparations. In the 1D-SDS-PAGE it was possible to observe the lower number of proteins in avium PPD preparation comparing to bovine PPD (data not shown) and no strong and

clear bands were observed in the preparation. We expected that the LC-MS/MS technique would identify a higher number of proteins in PPD preparations. However, it is conceivable that most mycobacterial proteins are extensively denatured by the preparation procedures of PPD, which explains the previous difficulties in isolating defined constituents from PPD in analyses by 1D or 2D-SDS-PAGE [12,19].

Comparing to previous studies, a great number of proteins was identified in this study. Of special interest is a group of 28 proteins (Rv0242c, Rv0248c, Rv0270, Rv0384c, Rv0467, Rv0623c, Rv0831c, Rv0896, Rv1093, Rv1133c, RV1630, Rv1745c, Rv1855c, Rv1916, Rv1926c, Rv1980c, Rv2593c, Rv2626c, Rv2873, Rv3001c, Rv3045, Rv3117, Rv3417c, RV3458c, Rv3716c, Rv3841, Rv3846, Rv3874) that are present in both bovine PPD preparations, but not in avium preparations. They are important because exposure of the animal to environmental mycobacteria can result in a positive skin test due to the presence of antigens shared with these species. The use of a PPD composed exclusively with antigens absent in environmental mycobacterial species would allow an improvement in the specificity of the test. Antigens that are absent in the BCG strain are also of great interest, as their use could result in a test able to differentiate infected from vaccinated animals. Some proteins such as MPB59, MPB64, MPB70, MPB83, ESAT 6 and CFP-10 have been tested in differential diagnostic tests as recombinant proteins or/and synthetic peptides, but only ESAT 6 and CFP10 are promising candidates as diagnostic reagents suitable to differentiating BCG-vaccinated animals from those infected with bovine tuberculosis [13-16]. Five proteins identified in PPD preparations (Rv1980c, Rv1984c, Rv2346c, Rv3874, Rv3875) are absent in *M. bovis* BCG vaccinal strain, therefore they could be used in a differential diagnostic test. However, the Rv2346c and Rv3874 were identified in avium PPD samples and for this, they are not good targets because of cross-reactivity with environmental mycobacteria [23].

A noticeable absence in our study is a protein named DPPD, which has been isolated from PPD preparations and used as a single antigen in skin tests with encouraging results [24,25]. A possible explanation for this fact is that there is an error in the annotation of the genome of several members of the *M. tuberculosis* complex. The annotated coding sequence uses an alternative start

code resulting in a distinct amino acid sequence for the Rv0061, the coding sequence for the DPPD protein. This way, the MASCOT software does not find a hit when analysing a peptide sequence from DPPD.

In conclusion, this study gives a comprehensive proteomic analysis of the tuberculin purified protein derivative components. We identified several proteins in five different preparations, which are potential targets to be used in the development of diagnostic reagents. We are currently evaluating selected recombinant antigens regarding their potential in skin tests for the diagnosis of tuberculosis.

5. ACKNOWLEDGMENTS

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6. REFERENCES

1. WHO. Global Tuberculosis Control. WHO report. WHO/CDS/TB/2004. 2004.

2. Daborn CJ, Grange JM. HIV/AIDS and its implications for the control of animal tuberculosis. Br Vet J 1993;**149:**405-17.

3. Bloom, B. R. and Fine, P. E. M. The BCG experience: implications for future vaccines against tuberculosis. in: B.R> Bloom (ed), Tuberculosis: pathogenicity, protection and control.American Society for Mycrobiology , 1994,531-557..

4. Buddle BM, de Lisle GW, Pfeffer A, Aldwell FE. Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with a low dose of BCG. Vaccine 1995;**13**:1123-30.

5. Buddle BM, Keen D, Thomson A, et al. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium vaccae*. Res Vet Sci 1995**;59**:10-6.

6. O'Reilly LM, Daborn CJ. The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. Tuber Lung Dis 1995;**76** Suppl 1:1-46.

7. Young DB. Heat-shock proteins: immunity and autoimmunity. Curr Opin Immunol 1992;**4**:396-400.

8. Daniel TM, Janicki BW. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol Rev 1978;**42**:84-113.

9. Dall'Stella R, Krieger MA, Burger M, et al. Development of bioprocess for the production of purified protein derivative with Brazilian strains of *Mycobacterium tuberculosis* for diagnosis use. J Biotechnol 2007;**127**:278-87.

10. Klausen J, Magnusson M, Andersen AB, Koch C. Characterization of purified protein derivative of tuberculin by use of monoclonal antibodies: isolation of a delayed-type hypersensitivity reactive component from M. tuberculosis culture filtrate. Scand J Immunol 1994;**40**:345-9.

11. Huebner RE, Schein MF, Bass JB, Jr. The tuberculin skin test. Clin Infect Dis 1993;**17**:968-75.

12. Rowland SS, Ruckert JL, Cummings PJ. Low molecular mass protein patterns in mycobacterial culture filtrates and purified protein derivatives. FEMS Immunol Med Microbiol 1999;**23**:21-5.

13. Buddle BM, Parlane NA, Keen DL, et al. Differentiation between Mycobacterium bovis BCG-vaccinated and M. bovis-infected cattle by using recombinant mycobacterial antigens. Clin Diagn Lab Immunol 1999;**6**:1-5.

14. Buddle BM, Ryan TJ, Pollock JM, et al. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Vet Microbiol 2001;**80**:37-46.

15. Vordermeier HM, Cockle PC, Whelan A, et al. Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and M. bovis infection in cattle. Clin Diagn Lab Immunol 1999;**6**:675-82.

16. Vordermeier HM, Whelan A, Cockle PJ, et al. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diagn Lab Immunol 2001;**8**:571-8.

17. Affronti LF, Lind A, Ouchterlony O, et al. An evaluation of the polyacrylamide gel electrophoresis fractionation method for the production of *Mycobacterium tuberculosis* skin test preparations. I. Production, physiochemical characterization and serological analyses. J Biol Stand 1986;**14**:177-86.

18. Bardarov SS, Kriakov J, Karakashyan A, et al. Characterization of PPD protein antigens in whole cell lysates of *Mycobacterium bovis* BCG. FEMS Microbiol Lett 1990;**59**:89-93.

19. Kitaura H, Kinomoto M, Yamada T. Ribosomal protein L7 included in tuberculin purified protein derivative (PPD) is a major heat-resistant protein inducing strong delayed-type hypersensitivity. Scand J Immunol 1999;**50**:580-7.

20. Hewinson RG, Vordermeier HM, Buddle BM. Use of the bovine model of tuberculosis for the development of improved vaccines and diagnostics. Tuberculosis (Edinb) 2003;**83**:119-30.

21. Palmer MV, Waters WR, Thacker TC, et al. Effects of different tuberculin skin-testing regimens on gamma interferon and antibody responses in cattle experimentally infected with *Mycobacterium bovis*. Clin Vaccine Immunol 2006;**13:**387-94.

22. Denis M, Wedlock DN, McCarthy AR, et al. Enhancement of the sensitivity of the whole-blood gamma interferon assay for diagnosis of *Mycobacterium bovis* infections in cattle. Clin Vaccine Immunol 2008;**14**:1483-9.

23. Hope JC, Thom ML, Villarreal-Ramos B, et al. Exposure to Mycobacterium avium induces low-level protection from *Mycobacterium*

bovis infection but compromises diagnosis of disease in cattle. Clin Exp Immunol 2005;**141**:432-9.

24. Campos-Neto A, Rodrigues-Junior V, Pedral-Sampaio DB, et al. Evaluation of DPPD, a single recombinant *Mycobacterium tuberculosis* protein as an alternative antigen for the Mantoux test. Tuberculosis (Edinb) 2001;**81**:353-8.

25. Coler RN, Skeiky YA, Ovendale PJ, et al. Cloning of a *Mycobacterium tuberculosis* gene encoding a purifed protein derivative protein that elicits strong tuberculosis-specific delayed-type hypersensitivity. J Infect Dis 2000;**182:**224-33.

Table 1. List of PPD proteins identified by LC-MS/MS

Rv number	Gene Name	Molecular mass(kDa)	Funcional group[a]	Protein Class[b]	BCG DEL[c]	PPD B BR	PPD B UK	PPD A BR1	PPD A BR2	PPD A UK	Protein identity
Rv0053	*rpsF*	10928	2	C				x			30S ribosomal protein S6
Rv0054	*ssb*	17400	2	C		x					single strand binding protein
Rv0125	*pepA*	37326	7	SIGNALP		x	x				probable serine protease
Rv0129c	*fbpC2*	36791	1	TATP			x	x	x	x	antigen 85C, mycolytransferase
Rv0216	*Rv0216*	35994	5,7	C		x	x	x	x	x	conserved hypothetical protein
Rv0242c	*fabG4*	46916	1	C		x	x				3-oxoacyl-[ACP] reductase
Rv0243	*fadA2*	46307	1	C			x				acetyl-CoA C-acetyltransferase
Rv0248c	*Rv0248c*	71092	7	C		x	x				probable flavoprotein subunit of Rv0247c
Rv0270	*fadD2*	59856	1	C		x	x				acyl-CoA synthase
Rv0281	*Rv0281*	33092	5, 10	C					x		conserved hypothetical protein
Rv0333	*Rv0333*	13066	6	C			x				hypothetical protein
Rv0350	*dnaK*	66659	0	C		x	x	x	x	x	70 kD heat shock protein, chromosome replication
Rv0351	*grpE*	21294	0	C			x				stimulates DnaK ATPase activity
Rv0379	*sec*	7961	3	C			x				probable transport protein SecE/Sec61- g family
Rv0384c	*clpB*	95767	0	C		*x*	*x*				heat shock protein
Rv0404	*fadD30*	64059	1	C				x			acyl-CoA synthase
Rv0431	*Rv0431*	16855	3	C			x				tuberculin related peptide (AT103)
Rv0440	*groEL2*	56561	0	C		x	x	x	x	x	60 kD chaperonin 2
Rv0455c	*Rv0455c*	19766	5, 10	SIGNALP					x	x	hypothetical protein
Rv0458	*Rv0458*	54825	7	C		x					aldehyde dehydrogenase
Rv0462	*lpd*	49437	7	C			x				probable dihydrolipoamide dehydrogenase
Rv0467	*aceA*	47228	7	C		x	x				isocitrate lyase
Rv0468	*fadB2*	30766	1	C		x					3-hydroxyacyl-CoA dehydrogenase
Rv0475	*Rv0475*	21391	3	C			x				possible exported protein
Rv0500	*proC*	30139	7	C		x					pyrroline-5-carboxylate reductase
Rv0503c	*cmaA2*	37359	1	C			x				cyclopropane mycolic acid synthase 2
Rv0569	*Rv0569*	14372	5, 10	C			x				conserved hypothetical protein
Rv0577	*Rv0577*	27251	5, 10	C			x				conserved hypothetical protein
Rv0583c	*lpqN*	23725	3	SIGNALP			x				equivalent to MKU20446_1 MK35
Rv0589	*mce2*	43576	0	C		x					cell invasion protein
Rv0632c	*echA3*	24454	1	C		x	x				enoyl-CoA hydratase/isomerase superfamily
Rv0639	*nusG*	25431	2	C			x				transcription antitermination protein

Rv0652	*rplL*	13432	2	C	x	x	x	x	x	50S ribosomal protein L7/L12
Rv0685	*tuf*	43566	2	SIGNALP	x	x	x	x	x	elongation factor EF-Tu
Rv0701	*rplC*	23133	2	C		x				50S ribosomal protein L3
Rv0703	*rplW*	10951	2	C		x				50S ribosomal protein L23
Rv0706	*rplV*	20368	2	C		x				50S ribosomal protein L22
Rv0707	*rpsC*	29928	2	C		x				30S ribosomal protein S3
Rv0733	*adk*	20113	7	C		x				probable adenylate kinase
Rv0761c	*adhB*	36283	7	C		x				zinc-containing alcohol dehydrogenase
Rv0801	*Rv0801*	12618	5, 10	C		x				hypothetical protein
Rv0828c	*Rv0828c*	16119	7	C			x	x		conserved hypothetical protein
Rv0831c	*Rv0831c*	33921	5, 10	C	x	x				conserved hypothetical protein
Rv0884c	*serC*	40266	7	C	x					phosphoserine aminotransferase
Rv0896	*gltA2*	48032	7	C	x	x				citrate synthase 1
Rv0905	*echA6*	26127	1	C		x				enoyl-CoA hydratase/isomerase superfamily
Rv0932c	*pstS*	38110	3	SIGNALP		x				PstS component of phosphate uptake
Rv0984	*moaB2*	18618	7	C	x					molybdenum cofactor biosynthesis, protein B
Rv1038c	*Rv1038c*	11097	3, 5	C		x			x	conserved hypothetical protein
Rv1070c	*echA8*	27370	1	C	x					enoyl-CoA hydratase/isomerase superfamily
Rv1080c	*greA*	17844	2	SECRETOMEP	x					transcription elongation factor G
Rv1093	*glyA*	45058	7	C	x	x				serine hydroxymethyltransferase
Rv1133c	*metE*	81816	7	C	x	x				5-methyltetrahydropteroyltriglutamate-homocystein
Rv1174c	*Rv1174c*	10988	3	SIGNALP		x				hypothetical protein
Rv1198	*Rv1198*	9979	3, 5	C		x				conserved hypothetical protein
Rv1270c	*lprA*	24973	3	SIGNALP		x				lipoprotein
Rv1324	*Rv1324*	32208	7	C		xx				hypothetical protein
Rv1352	*Rv1352*	12955	5, 10	SIGNALP		x				conserved hypothetical protein
Rv1388	*mIHF*	12097	2	C		x	x		x	integration host factor
Rv1392	*metK*	43447	7	C		x				S-adenosylmethionine synthase
Rv1411c	*lprG*	35588	3	SIGNALP		x				lipoprotein
Rv1436	*gap*	36105	7	C	x	x		x		glyceraldehyde 3-phosphate dehydrogenase
Rv1448c	*tal*	40723	7	C	x	x		x		transaldolase
Rv1475c	*acn*	102728	7	C				x		aconitate hydratase
Rv1596	*nadC*	29859	7	C			x			nicotinate-nucleotide pyrophosphatase
Rv1608c	*bcpB*	16998	0	C					x	probable bacterioferritin comigratory protein
Rv1630	*rpsA*	53199	2	C	x	x				30S ribosomal protein S1
Rv1636	*Rv1636*	15303	5, 10	C					x	conserved hypothetical protein
Rv1655	*argD*	41055	7	C				x	x	acetylornithine aminotransferase
Rv1687c	*Rv1687c*	27940	3	C				x		probable ABC transporter
Rv1702c	*Rv1702c*	49935	5	C				x		REP-family protein
Rv1745c	*Rv1745c*	102728	7	C	x	x				conserved hypothetical protein

Rv1793	*Rv1793*	9993	3, 5	C			x				conserved hypothetical protein
Rv1796	*Rv1796*	60275	7	TMHMM						x	conserved hypothetical protein
Rv1826	*gcvH*	14229	7	C			x				glycine cleavage system H protein
Rv1827	*Rv1827*	17240	5, 10	C		x	x	x	x	x	conserved hypothetical protein
Rv1837c	*glcB*	80450	7	C		x	x	x	x		malate synthase
Rv1855c	*Rv1855c*	33256	7	C		x	x				probable monooxygenase
Rv1860	*modD*	28746	3	SIGNALP			x	x		x	precursor of Apa (45/47 kD secreted protein)
Rv1876	*bfrA*	18443	7	C		x	x		x	x	bacterioferritin
Rv1886c	*fbpB*	30814	1	TATP			x				antigen 85B, mycolyltransferase
Rv1893	*Rv1893*	7463	5, 10	C			x				hypothetical protein
Rv1916	*aceAb*	85437	7	C		x	x				isocitrate lyase, [beta] module
Rv1926c	*Rv1926c*	16504	3	SIGNALP		x	x				hypothetical protein
Rv1932	*tpx*	17057	0	C			x				thiol peroxidase
Rv1980c	*mpt64*	25081	3	SIGNALP	x	x	x				secreted immunogenic protein Mpb64/Mpt64
Rv1984c	*Rv1984c*	24210	3	SIGNALP	x		x				probable secreted protein
Rv2000	*Rv2000*	61142	6, 16	C					x	x	hypothetical protein
Rv2031c	*hspX*	16086	0	C			x				14kD antigen, heat shock protein Hsp20 family
Rv2140c	*TB18.6*	18622	5, 10	C			x				conserved hypothetical protein
Rv2145c	*wag31*	28260	3	C		x	x		x	x	antigen 84 (aka wag31)
Rv2215	*sucB*	57110	7	C			x				dihydrolipoamide succinyltransferase
Rv2217	*lipB*	25646	7	C				x			lipoate biosynthesis protein B
Rv2220	*glnA1*	53707	7	C			x				glutamine synthase class I
Rv2241	*aceE*	100481	7	C			x				pyruvate dehydrogenase E1 component
Rv2244	*acpM*	12516	1	C		x	x	x	x	x	acyl carrier protein (meromycolate extension)
Rv2246	*kasB*	44421	1	C			x				[beta]-ketoacyl-ACP synthase (meromycolate
Rv2339	*mmpL9*	90186	3	TMHMM				x			conserved large membrane protein
Rv2346c	*Rv2346c*	6315	3	C	x					x	conserved hypothetical protein
Rv2363	*amiA2*	51023	7	C				x		x	putative amidase
Rv2376c	*Rv2376c*	16653	3	SIGNALP			x				conserved hypothetical protein
Rv2427c	*proA*	43832	7	C		x					[gamma]-glutamyl phosphate reductase
Rv2428	*ahpC*	21724	0	C				x	x		alkyl hydroperoxide reductase
Rv2467	*pepD*	94683	7	C					x		probable aminopeptidase
Rv2557	*Rv2557*	24679	5, 16	C			x				conserved hypothetical protein
Rv2593c	*ruvA*	20177	2	C		x	x				Holliday junction binding protein, DNA helicase
Rv2623	*Rv2623*	31747	3	C			x				conserved hypothetical protein
Rv2626c	*Rv2626c*	15679	5, 10	C		x	x				conserved hypothetical protein
Rv2704	*Rv2704*	14619	5, 10	C				x			conserved hypothetical protein
Rv2837c	*Rv2837c*	35621	5, 10	C				x			conserved hypothetical protein
Rv2873	*mpt83*	24412	3	SIGNALP		x	x				surface lipoprotein Mpt83

Rv2875	*mpt70*	8674	3	SIGNALP		x	x	x		x	major secreted immunogenic protein Mpt70
Rv2878c	*mpt53*	18959	3	SIGNALP			x				secreted protein Mpt53
Rv2882c	*frr*	20815	2	C						x	ribosome recycling factor
Rv2889c	*tsf*	28851	2	C		x					elongation factor EF-Ts
Rv2919c	*glnB*	12220	9	C						x	nitrogen regulatory protein
Rv2943	*Rv2943*	46262	5	C				x			hypothetical protein
Rv2945c	*lppX*	24296	3	SIGNALP			x				lipoprotein
Rv3001c	*ilvC*	36626	7	C		x	x				ketol-acid reductoisomerase
Rv3028c	*fixB*	31700	7	C		x	x		x		electron transfer flavoprotein [alpha] subunit
Rv3036c	*Rv3036c*	24590	3	SIGNALP			x				probable secreted protein
Rv3045	*adhC*	37508	7	C		x	x				alcohol dehydrogenase
Rv3046c	*Rv3046c*	13375	5, 10	C			x				conserved hypothetical protein
Rv3048c	*nrdG*	37025	2	C			x				ribonucleoside-diphosphate small subunit
Rv3075c	*Rv3075c*	33194	5, 10	C		x					conserved hypothetical protein
Rv3117	*cysA3*	31110	7	C		x	x				thiosulfate sulfurtransferase
Rv3244c	*lpqB*	24897	3	SIGNALP						x	lipoprotein
Rv3248c	*sahH*	54343	7	C		x	x	x	x		adenosylhomocysteinase
Rv3354	*Rv3354*	13065	5, 10	SIGNALP			x				conserved hypothetical protein
Rv3389c	*Rv3389c*	30522	7	C		x					putative dehydrogenase
Rv3417c	*groEL1*	55858	0	C		x	x				60 kD chaperonin 1
Rv3418c	*groES*	10798	0	C		x	x	x	x	x	10 kD chaperone
Rv3443c	*rplM*	16327	2	C		x					50S ribosomal protein L13
Rv3456c	*rplQ*	19493	2	C		x					50S ribosomal protein L17
Rv3457c	*rpoA*	37740	2	C			x				[alpha] subunit of RNA polymerase
Rv3458c	*rpsD*	23330	2	C		x	x				30S ribosomal protein S4
Rv3615c	*Rv3615c*	10845	5, 10	C			x				conserved hypothetical protein
Rv3663c	*dppD*	59079	3	C		x	x	x	x	x	probable ABC-transporter
Rv3716c	*Rv3716c*	13349	5, 10	C		x	x				conserved hypothetical protein
Rv3722c	*Rv3722c*	44460	5, 10	C			x				hypothetical protein
Rv3797	*fadE35*	65681	1	C			x				acyl-CoA dehydrogenase
Rv3804c	*fbpA*	37977	1	TATP			x	x	x		antigen 85A, mycolyltransferase
Rv3841	*bfrB*	20429	7	C		x	x				bacterioferritin
Rv3846	*sodA*	22044	0	C		x	x				superoxide dismutase
Rv3874	*esxB*	10656	3	C	x	x	x			x	conserved hypothetical protein
Rv3875	*esat6*	9767	3	C	x		x				early secreted antigen target 6
Rv3914	*trxC*	12520	7	C			x				thioredoxin

[a] Functional groups: (0) virulence, detoxification, adaptation; (1) lipid metabolism; (2) information pathways; (3) cell wall and cell processes; (4) stable RNAs; (5) insertion seqs and phages; (6) PE/PPE; (7) intermediary metabolism and respiration; (8) unknown; (9) regulatory proteins; (10) conserved hypothetical; (16) conserved hypothetical with an orthologue in *M. bovis*. [b]Protein class: (C) cytoplasm protein; (TMHMM) membrane protein; (TATP) Tat signal peptide; (SIGNALP) Sec signal

peptide; (SECRETOMEP) Non-classical secretion. [c]Genes deleted in the *M. bovis* BCG strain.

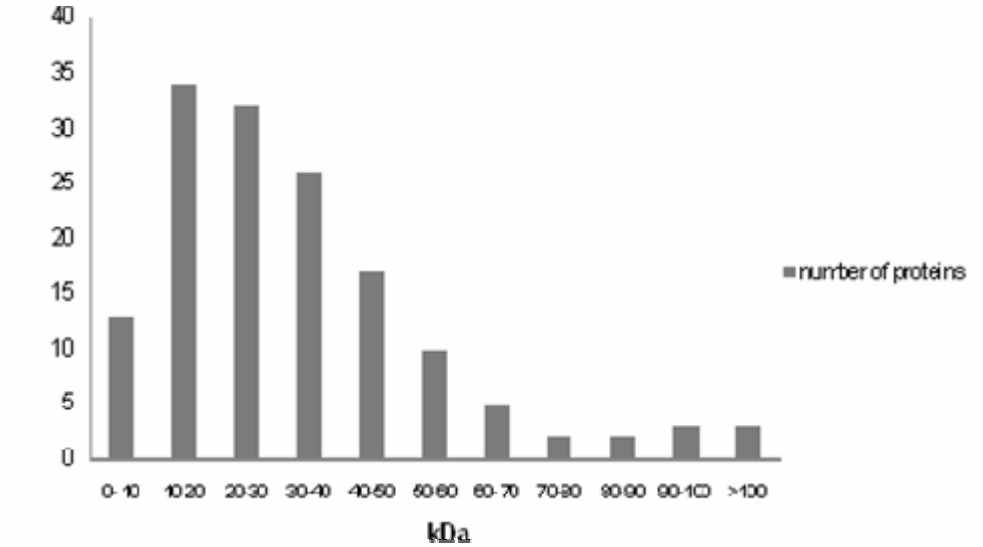
Table 2. List of proteins with higher number of peptides identified by LC-MS/MS. Shared proteins are in bold. The one shared in all samples is in bold and underline.

PPD Bov BR		PPD Bov UK		PPD Avi BR 1		PPD Avi BR 2		PPD Avi UK	
Protein	**N° peptides[a]**	**Protein**	**N° peptides**	**Protein**	**N° peptides**	**Protein**	**N° peptides**	**Protein**	**N° peptides**
Rv3663c	187	**Rv3874**	188	**Rv3663c**	103	**Rv3663c**	199	**Rv3663c**	88
Rv0685	122	**Rv2875**	133	Rv3428c	66	**Rv0652**	95	**Rv0652**	45
Rv0350	112	**Rv0440**	132	**Rv0652**	44	**Rv3248c**	40	**Rv2244**	36
Rv1133c	108	**Rv0350**	102	**Rv0685**	41	**Rv0350**	33	Rv3418c	33
Rv0440	80	**Rv3663c**	94	**Rv3804c**	21	**Rv3804c**	33	Rv1860	24
Rv3001c	61	Rv3418c	92	**Rv0350**	18	**Rv0685**	27	**Rv0440**	20
Rv1916	56	Rv2873	68	**Rv3248c**	17	**Rv0440**	26	Rv1876	19
Rv0467	43	Rv2031c	66	Rv0629c	17	Rv0129c	20	Rv0455c	16
Rv0216	40	**Rv2244**	63	**Rv2244**	17	Rv2744	18	**Rv3874**	12
Rv2875	41	Rv3875	62	**Rv0440**	15	Rv1876	18	**Rv0685**	11

FIGURE CAPTIONS

Figure 1. Distribution of molecular mass of the identified PPD proteins by LC-MS/MS.

Borsuk et al. Figure 1.



5 ARTIGO 4

THE KNOCKOUT OF THE DPPD-Mb0092 OF *Mycobacterium bovis* BCG MAY CONTRIBUTE TO THE DEVELOPMENT OF A SKIN TEST ABLE TO DIFFERENTIATE BCG VACCINATED AND INFECTED INDIVIDUALS

(Artigo a ser submetido ao periódico *FEMS Mycrobiology Letters*)

THE KNOCKOUT OF THE DPPD-Mb0092 OF *Mycobacterium bovis* BCG MAY CONTRIBUTE TO THE DEVELOPMENT OF A SKIN TEST ABLE TO DIFFERENTIATE BCG VACCINATED AND INFECTED INDIVIDUALS

Sibele Borsuk[a]; Jane Newcombe[b], Tom Mendum[b], Odir Antônio Dellagostin[a] , Johnjoe McFadden[b]

[a] *Centro de Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil,*

[b] *School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, UK.*

[*] Corresponding author

Centro de Biotecnologia, Universidade Federal de Pelotas. CP 354, 96010-900, Pelotas, RS, Brazil, Phone: +55 53 32757350. E-mail: odirad@terra.com.br.

ABSTRACT

Development of diagnostic tests which can readily differentiate between vaccinated and tuberculosis-infected individuals is crucial for the wider utilization of BCG as vaccine in humans and animals. DPPD is an antigen that elicits specific delayed type hypersensitivity reactions similar in size and morphological aspects to that elicited by purified protein derivative, in both animals and humans infected with the tubercle bacilli. We carried out a knockout of the gene that encodes this antigen in *Mycobacterium bovis* BCG by homologous recombination. The flanking sequences of the target gene (Mb0092) were cloned into a suicide vector. Spontaneous double crossovers, which result in wild type revertants or knockouts, were selected using SacB. The knockout genotype was confirmed by PCR and by Southern blot. This mutant BCG strain has the potential of inducing protection against tuberculosis without interfering with the skin test used for diagnosis, when performed using recombinant DPPD.

Key words: gene knockout, DPPD, tuberculosis diagnosis

1. INTRODUCTION

Tuberculosis in humans and animals continues to cause major health problems on a global scale (WHO, 2002). Bovine tuberculosis is an important cause of economic loss and can be a zoonotic infection (Daborn & Grange, 1993). An important control strategy for the prevention of these diseases is the use of effective vaccines. The *M. bovis* bacillus Calmette-Guerin (BCG) vaccine has been widely used for control of human tuberculosis (Bloom & Fine, 1994). In cattle, BCG has been used in a series of trials; with various degrees of protection against *M. bovis* challenge (Buddle *et al.*, 1995a; Buddle *et al.*, 1995b; O'Reilly & Daborn, 1995). Therefore, a major constraint in the use of attenuated mycobacterial vaccines such as BCG is that vaccination of humans or cattle interferes with the tuberculin skin test (Mantoux), a diagnostic test that has been used for more than 85 years (Huebner *et al.*, 1993). However, the test lacks specificity for *M. tuberculosis/bovis* due to the following factors: 1) exposure to or infection with mycobacteria other than *M. tuberculosis/bovis;* 2) vaccination with BCG; and 3) presence in the purified protein derivative (PPD) of several antigens shared by all *Mycobacterium (Young et al., 1992)*. Tests based on PPD cannot distinguish between tuberculosis infection, *Mycobacterium bovis* BCG vaccination or exposure to environmental mycobacteria**.** The development of diagnostic tests which can differentiate vaccinated and tuberculosis-infected individuals could greatly assist in the diagnosis of early infection as well as enhance the use of tuberculosis vaccines on a wider scale.

Alternatives to skin testing have been investigated. A diagnostic test for *M. bovis* infection in cattle has been developed. Detection of gamma-interferon (IFNγ) by sandwich ELISA has been tested (Dalley *et al.*, 2007). Recombinant proteins MPB59, MPB64, MPB70 and ESAT 6 also have been tested in differential diagnostic tests based on IFNγ detection (Buddle *et al.*, 1999; Buddle *et al.*, 2001). Molecular detection system by means of a real-time based PCR was tested (Parra *et al.*, 2007). However, for a test to be implemented, the low cost and easiness of execution is important.

A recombinant *M. tuberculosis* protein present in PPD that elicits strong and tuberculosis specific delayed type hypersensitivity (DTH) in guinea pigs has

been identified and characterized (Coler *et al.*, 2000). The DPPD protein is present only in tuberculosis complex mycobacteria and elicited DTH reactions in tuberculosis patients comparable to those elicited by tuberculin (Campos-Neto *et al.*, 2001). The results suggest that this molecule can be an additional tool for a more specific diagnosis of tuberculosis in non-BCG vaccinated individuals (Campos-Neto *et al.*, 2001), however, it does not alleviate the problem with BCG vaccinated individuals.

A BCG strain that does not produce DPPD would allow the use of this single recombinant antigen as a tool for the diagnosis of tuberculosis infection even in BCG vaccinated individuals. BCG vaccination could then be carried out in animals, without interfering with this new skin test. In this work we performed knockout of the *dppd* gene by homologous recombination. This new BCG Δ*dppd* strain will be characterized regarding its immunologic and protective properties comparing to the wild type strain.

2. MATERIALS AND METHODS

M. bovis BCG Pasteur strain 1173P2 was used as a source of DNA and as the parent strain for deletion of the *dppd* gene (GenBank accession number NC_008769, locus_tag BCG_0092) by homologous recombination. The strain was cultured in Middlebrook 7H9 (Difco) liquid medium supplemented with 10% of oleic acid albumin/dextrose complex (OADC; Difco), 0.05% Tween 80, and Middlebrook 7H11 (Difco) solid medium supplemented with 10% OADC. Recombinants were selected on 7H11 medium supplemented with 20 μg/mL kanamycin, 100 μg/mL X-Gal and 10% of sucrose when necessary.

Unmarked BCG Δ*dppd* was constructed as described by Parish and Stoker.[21] This strategy involves PCR amplifying chromosomal regions from either side of the gene of interest and cloning them into a suicide vector containing kanamycin resistance, *lacZ* and the counter selective *sacB* gene. When transformed into mycobacteria the plasmid integrates into the chromosome by a single cross-over to give $LacZ^{+}$, Kan^{R} colonies. Spontaneous double crossovers, which result in wild type revertants or knock-outs, can then be selected by using *SacB*.

The homologous chromosomal regions were PCR amplified from regions upstream (0091F- CGAGAAGCTTGAAGTTACCATCGGCAAGA and 0091R-ACTAGGATCCCCTTAACGACGAGCCGGT) and downstream (0093F-AATAGGATCCGCCGCGGCAATGAGT 0093R-CATCTTAATTAATTGAGCCTGGCGGCCATT) of the BCG *Pasteur.* These were cloned into p2-NIL using *Hin*dIII/B*am*HI and *Bam*HI/*Pac*I restriction sites. The resulting plasmid was ligated to the pGOAL-19 *Pac*I fragment (Parish & Stoker, 2000).

To transform the knockout plasmids into mycobacteria, cultures in logarithmic growth at OD_{600} of 0.6-1, were harvested by centrifugation. The pellet was washed in ×1, ×0.5 and ×0.2 volumes of 10% glycerol (37 °C for BCG) and the cells finally resuspended in ×0.01 volume. A 200 μl aliquot of competent cells were transformed by electroporation (25 μF, 1000 Ω, 2.5 V) with approximately 1 μg UV irradiated (100 mJ/cm^2) plasmid. BCG cells were recovered in 10 ml 7H9 for 12-24 h and plated on 7H11 with kanamycin and X-Gal. Colonies with β-galactosidase activity were sub-cultured onto plates containing X-Gal and 10% sucrose. White colonies were selected and their genotype determined by PCR using 0091-F and 0093-R, and by Southern blot using probes containing the homologous regions, the *dppd* gene and pGOAL-19.

3. RESULTS AND DISCUSSION

The strategy used for knocking out the Mb0092 open reading frame, which codes for the DPPD antigen is shown in the figure 1. The sequences upstream and downstream of the target gene were cloned into a suicide vector. The plasmid containing the sequences that flank the *dppd* gene was named pDppd. Unmarked *M. bovis* BCG *Δdppd* was successfully constructed by homologous recombination using the strategy described by Parish and Stoker (Parish & Stoker, 2000). The knockout genotype was confirmed by PCR using primers that anneal to a sequence flanking the knockout target. The knockout genotype allowed amplification of fragment of 1,822 bp, while the wild BCG resulted in the amplification of a 2,248 bp fragment (figure 2). PCR using the *dppd* gene as

target resulted in no amplification, further confirming the knockout genotype data not shown). Southern blot after probing with *dppd* gene and pGOAL-19 also resulted in no hybridization confirming the knockout genotype (data not shown).

Diagnosis of tuberculosis, particularly in countries where BCG is not used, as well as in farm animals, relies mainly on the tuberculin skin test, a test with limited specificity due to the presence in the PPD of antigens common to pathogenic as well as environmental species of mycobacteria. The possibility of using DPPD, a single antigen in the skin test instead of a complex mixture of antigens, reported by Campos-Neto et al. (2001) is very attractive, mainly due to the fact that this antigen is not present in environmental mycobacteria species. However, when the test is performed in BCG vaccinated individuals, a positive result does not have any clinical significance. In cattle, current tuberculosis is entirely based on the tuberculin skin test to identify infected animals and the subsequent slaughter of such tuberculin-positive animals. For this reason, BCG vaccination is not allowed in this species.

The encouraging results obtained with use of DPPD in the skin test, with similar results in terms of sensitivity and improved specificity (Campos-Neto et al., 2001) prompted us to investigate the possibility of genetically modify the BCG strain in order to knock out this antigen. The method used for generating unmarked mutation by homologous recombination was very successful. In the first attempt we obtained colonies with the *dppd* gene knock-out. This BCG Δ*dppd* strain will be evaluated in animal models of tuberculosis to ascertain that the protective properties of the BCG vaccine have not been compromised. It is expected that this mutant strain will be able to protect against the severe forms of tuberculosis without sensitizing the animal against the DPPD antigen. If this hypothesis is confirmed, a new perspective for vaccination of farm animals with BCG will be established. This will provide not only protection against tuberculosis (Wiker HG *et al.*, 2006), but also the possibility of using BCG as a multivalent vaccine vector.

4. REFERENCES

Bloom BR & Fine PEM. The BCG experience: implications for future vaccines against tuberculosis. in: B.R> Bloom (ed), Tuberculosis: pathogenicity, protection and control.American Society for Mycrobiology , 531-557. 1994.

Buddle BM, de Lisle GW, Pfeffer A, & Aldwell FE (1995a). Immunological responses and protection against Mycobacterium bovis in calves vaccinated with a low dose of BCG. *Vaccine* **13**, 1123-1130.

Buddle BM, Keen D, Thomson A, Jowett G, McCarthy AR, Heslop J, de Lisle GW, Stanford JL, & Aldwell FE (1995b). Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed Mycobacterium vaccae. *Res Vet Sci* **59**, 10-16.

Buddle BM, Parlane NA, Keen DL, Aldwell FE, Pollock JM, Lightbody K, & Andersen P (1999). Differentiation between Mycobacterium bovis BCG-vaccinated and M. bovis-infected cattle by using recombinant mycobacterial antigens. *Clin Diagn Lab Immunol* **6**, 1-5.

Buddle BM, Ryan TJ, Pollock JM, Andersen P, & de Lisle GW (2001). Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. *Vet Microbiol* **80**, 37-46.

Campos-Neto A, Rodrigues-Junior V, Pedral-Sampaio DB, Netto EM, Ovendale PJ, Coler RN, Skeiky YA, Badaro R, & Reed SG (2001). Evaluation of DPPD, a single recombinant Mycobacterium tuberculosis protein as an alternative antigen for the Mantoux test. *Tuberculosis (Edinb)* **81**, 353-358.

Coler RN, Skeiky YA, Ovendale PJ, Vedvick TS, Gervassi L, Guderian J, Jen S, Reed SG, & Campos-Neto A (2000). Cloning of a Mycobacterium tuberculosis

gene encoding a purifed protein derivative protein that elicits strong tuberculosis-specific delayed-type hypersensitivity. *J Infect Dis* **182**, 224-233.

Daborn CJ & Grange JM (1993). HIV/AIDS and its implications for the control of animal tuberculosis. *Br Vet J* **149**, 405-417.

Dalley D, Dave D, Lesellier S, Palmer S, Crawshaw T, Hewinson RG, & Chambers M (2007). Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (Meles meles). *Tuberculosis (Edinb)*.

Huebner RE, Schein MF, & Bass JB, Jr. (1993). The tuberculin skin test. *Clin Infect Dis* **17**, 968-975.

O'Reilly LM & Daborn CJ (1995). The epidemiology of Mycobacterium bovis infections in animals and man: a review. *Tuber Lung Dis* **76 Suppl 1**, 1-46.

Parish T & Stoker NG (2000). Use of a flexible cassette method togenerate a double unmarked *Mycobacterium tuberculosis tlyA plcABC* mutant by gene replacement. *Microbiology* **146**, 1969-1975.

Parra A, Garcia N, Garcia A, Lacombe A, Moreno F, Freire F, Moran J, & Hermoso de MJ (2007). Development of a molecular diagnostic test applied to experimental abattoir surveillance on bovine tuberculosis. *Vet Microbiol*.

WHO. Global Tuberculosis Control. WHO report. WHO/CDS/TB/2002. 2002. Ref Type: Data File

Wiker HG, Mustafa T, Målen H, & Riise AM (2006). Vaccine approaches to prevent tuberculosis. *Scand J Immunol* **64**, 243-250.

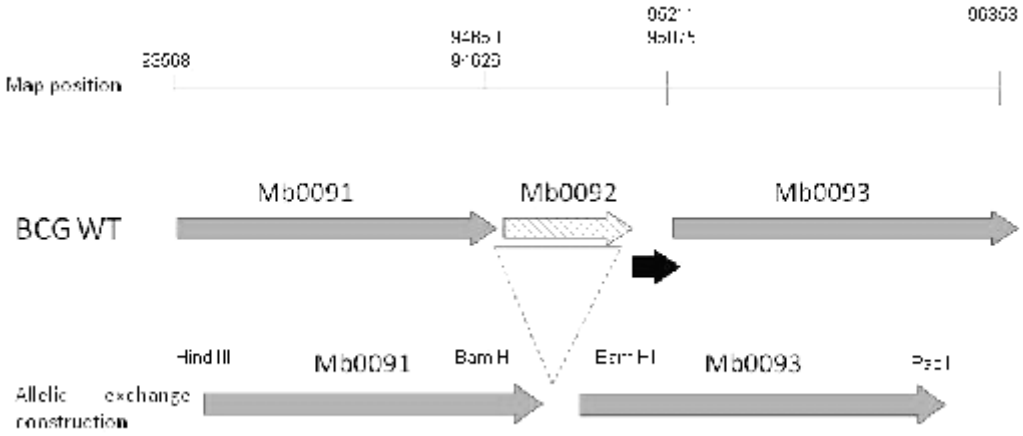
Young DB, Kaufmann SH, Hermans PW, & Thole JE (1992). Mycobacterial protein antigens: a compilation. *Mol Microbiol* **6**, 133-145.

FIGURE CAPTIONS

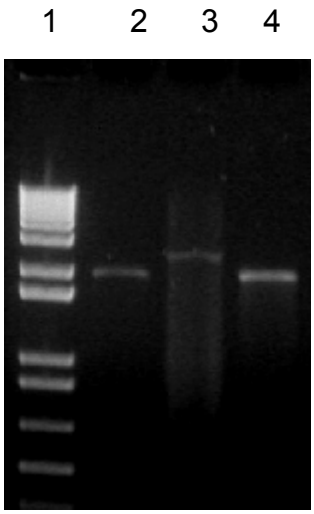
Figure. 1 Alignment of the *M. tuberculosis* genome at the dppd locus (Mb0092) with the DNA construct used for dppd knockout by homologous recombination.

Figure 2: Agarose gel electrophoresis of PCR products produced using primers flanking the *dppd* gene for: lane 1, 1 Kb DNA Ladder (Invitrogen), lane 2, the *dppd* knockout mutant -BCG Pasteur Δ*dppd* ; lane 3, BCG WT (negative control) ; lane 4, pDppd (positive control).

Borsuk et al. Figure 1



Borsuk et al. Figure 2



6 CONCLUSÕES

1. A seleção de BCG recombinante por complementação auxotrófica foi tão eficiente quanto à seleção por resistência a antibiótico, podendo ser utilizada em substituição a esta;

2. O sistema de complementação auxotrófica foi altamente estável *in vitro* e *in vivo*, enquanto o sistema convencional com a utilização de antibiótico foi instável;

3. Proteínas que compõe o PPD bovino e PPD aviário foram identificadas por LC-MS/MS, abrindo caminho para que estas sejam avaliadas individualmente ou em conjunto, na forma recombinante, visando o desenvolvimento de um novo reagente diagnóstico, com o potencial para diferenciar animal infectado de vacinado com BCG;

4. A cepa *M. bovis* BCG *Δdppd* foi construída, podendo ser útil na proteçao contra tubeculose sem interfir no diagnóstico.

7 REFERÊNCIAS

ABDELHAK S, LOUZIR H, TIMM J, BLEL L, BENLASFAR Z, LAGRANDERIE M, GHEORGHIU M, DELLAGI K, & GICQUEL B Recombinant BCG Expressing the Leishmania Surface-Antigen Gp63 Induces Protective Immunity Against Leishmania-Major Infection in Balb/C Mice. **Microbiology**, 141, 1585-1592, 1995.

BLOOM BR & FINE PEM. The BCG experience: implications for future vaccines against tuberculosis. in: B.R> Bloom (ed), Tuberculosis: pathogenicity, protection and control. **American Society for Mycrobiology** , 531-557. 1994.

BORSUK S, MENDUM TA, FAGUNDES MQ, MICHELON M, CUNHA CW, MCFADDEN J, & DELLAGOSTIN OA. Auxotrophic complementation as a selectable marker for stable expression of foreign antigens in *Mycobacterium bovis* BCG. **Tuberculosis** 87, 474-480, 2007.

BUDDLE BM, PARLANE NA, KEEN DL, ALDWELL FE, POLLOCK JM, LIGHTBODY K, & ANDERSEN P. Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. **Clinical Diagnostic Laboratorial Immunologycal** 6, 1-5, 1999.

BUDDLE BM, RYAN TJ, POLLOCK JM, ANDERSEN P, & DE LISLE GW. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. **Veteterinary Microbiology** 80, 37-46, 2001.

BUDDLE B, WEDLOCK DN, & DENIS M. Progress in the development of tuberculosis vaccines for cattle and wildlife. **Veterinary microbiology** 25, 191-200, 2006.

CHAMBERS MA, WILLIANS A, GAVIER-WIDE´N D, WHELAN A, HALL G, MARSH PD, BLOOM BR, JACOBS WR, & HEWINSON G . Identification of a *Mycobacterium bovis* BCG Auxotrophic Mutant That Protects Guinea Pigs against *M. bovis* and Hematogenous Spread of *Mycobacterium tuberculosis* without Sensitization to tuberculin. **Infection and Immunity** 68, 7094-7099, 2000.

DALL'STELLA R, KRIEGER MA, BURGER M, AGOTTANI JB, CHAHAD-EHLERS S, & THOMAZ-SOCCOL V. Development of bioprocess for the production of purified protein derivative with Brazilian strains of *Mycobacterium tuberculosis* for diagnosis use. **Journal of Biotechnology** 127, 278-287, 2007.

DALLEY D, DAVE D, LESELLIER S, PALMER S, CRAWSHAW T, HEWINSON RG, & CHAMBERS M. Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (*Meles meles*). **Tuberculosis**, 2007.

DANIEL TM & JANICKI BW (1978). Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. **Microbiological reviews 42**, 84-113, 1978.

DENNEHY M & WILLIAMSON AL. Factors influencing the immune response to foreign antigen expressed in recombinant BCG vaccines. **Vaccine** 23, 1209-1224, 2005.

DONNELLYWU MK, JACOBS WR, & HATFULL GF. Superinfection Immunity of Mycobacteriophage-L5 - Applications for Genetic-Transformation of Mycobacteria. **Molecular Microbiology** 7, 407-417, 1993.

GULERIA I, TEITELBAUM R, MCADAM RA, KALPANA G, JACOBS WR, & BLOOM BR . Auxotrophic vaccines for tuberculosis. **Nature Medicine** 2, 334-337, 1996.

HUEBNER RE, SCHEIN MF, & BASS JB, JR. The tuberculin skin test. *Clin* **Infectious diseases** 17, 968-975, 1993.

HUSSON RN, JAMES BE, & YOUNG RA (1990). Gene Replacement and Expression of Foreign DNA in Mycobacteria. **Journal of Bacteriology** 172, 519-524.

JACKSON NM, PHALEN SW, Lagranderie M, Ensergueix D, CHAVAROT P, MARCHAL G, MCMURRAY DN, Gicquel B, & Guilhot C. Persistence and Protective Efficacy of a *Mycobacterium tuberculosis* Auxotroph Vaccine. **Infection and Immunity 67**, 2867-2873, 1999.

JACOBS WR, TUCKMAN M, & BLOOM BR . Introduction of Foreign DNA Into Mycobacteria Using a shuttle phasmid. **Nature** 327, 532-535, 1987.

KLAUSEN J, MAGNUSSON M, ANDERSEN AB, & KOCH C (1994). Characterization of purified protein derivative of tuberculin by use of monoclonal antibodies: isolation of a delayed-type hypersensitivity reactive component from M. tuberculosis culture filtrate. **Scandinavian journal of immunology** 40, 345-349, 1994.

MATSUMOTO S, YUKITAKE H, KANBARA H, & YAMADA T . 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. **The Journal of experimental medicine** 188, 845-854, 1998.

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

MCADAM RA, WEISBROD TR, MARTIN J, SCUDERI JD, BROWN AM, CIRILLO JD, BLOOM BR, & JACOBS WR. In-Vivo Growth-Characteristics of Leucine and Methionine Auxotrophic Mutants of Mycobacterium-Bovis BCG Generated by Transposon Mutagenesis. **Infection and Immunity 63**, 1004-1012, 1995.

MEDEIROS MA, DELLAGOSTIN OA, ARMOA GRG, DEGRAVE WM, 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, 1999-2009.

MEISSNER PS & FALKINHAM JO (1984). Plasmid-Encoded Mercuric Reductase in Mycobacterium-Scrofulaceum. **Journal of Bacteriology** 157, 669-672, 1984.

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 LCC . Recombinant Mycobacterium bovis BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live Bordetella pertussis in mice. **Infection and Immunity** 68, 4877-4883, 2000.

O'DONNELL MA (1997). The genetic reconstruction of BCG as a new immunotherapeutic tool. **Trends Biotechnology** 15, 512-517.

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

PARRA A, GARCIA N, GARCIA A, LACOMBE A, MORENO F, FREIRE F, MORAN J, & HERMOSO DE MJ. Development of a molecular diagnostic test applied to experimental abattoir surveillance on bovine tuberculosis. **Veterinary Microbioogy** *in press***,** (2007)

PAVELKA MS, CHEN B, KELLEY CL, COLLINS FM, & JACOBS WR. Vaccine efficacy of a lysine auxotroph of Mycobacterium tuberculosis. **Infection and Immunity** 71, 4190-4192, 2003.

ROWLAND SS, RUCKERT JL, & CUMMINGS PJ. Low molecular mass protein patterns in mycobacterial culture filtrates and purified protein derivatives. **FEMS immunology and medical microbiology** 23, 21-25, 1999.

SEIXAS FK, SILVA EF, HARTWIG DD, CERQUEIRA GM, QUEVEDO M, DOSSA R, & DELLAGOSTIN OA. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. **Vaccine,** 26, 88-95**,** 2007.

SKJOT RL, OETTINGER T, ROSENKRANDS I, RAVN P, BROCK I, JACOBSEN S, & ANDERSEN P. Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. **Infection and Immunity** 68, 214-220, 2000.

SNAPPER SB, LUGOSI L, JEKKEL A, MELTON RE, KIESER T, BLOOM BR, & JACOBS WR. Lysogeny and Transformation in Mycobacteria - Stable Expression of Foreign Genes. **Proceedings of the National Academy of Sciences of the United States of America** 85, 6987-6991, 1988.

VAN PINXTEREN LA, RAVN P, AGGER EM, POLLOCK J, & ANDERSEN P. Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. **Clinical and diagnostic laboratory immunology** 7, 155-160, 2000.

YOUNG DB. Heat-shock proteins: immunity and autoimmunity. **Current opinion in immunology** 4, 396-400, 1992.

8 ANEXOS

Artigo 2: Publicado no Periódico **Tuberculosis**

Tuberculosis (2007) **87**, 474–480

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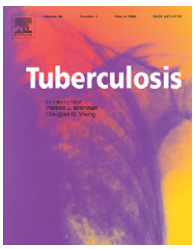

Auxotrophic complementation as a selectable marker for stable expression of foreign antigens in *Mycobacterium bovis* BCG

Sibele Borsuk[a], Tom A. Mendum[b], Michel Quevedo Fagundes[a], Marcelo Michelon[a], Cristina Wetzel Cunha[a], Johnjoe McFadden[b], Odir Antônio Dellagostin[a,*]

[a]*Centro de Biotecnologia, Universidade Federal de Pelotas, CP-354, 96010-900 Pelotas, RS, Brazil*
[b]*School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, UK*

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Summary
Mycobacterium bovis BCG has the potential to be an effective live vector for multivalent vaccines. However, most mycobacterial cloning vectors rely on antibiotic resistance genes as selectable markers, which would be undesirable in any practical vaccine. Here we report the use of auxotrophic complementation as a selectable marker that would be suitable for use in a recombinant vaccine. A BCG auxotrophic for the amino acid leucine was constructed by knocking out the *leuD* gene by unmarked homologous recombination. Expression of *leuD* on a plasmid not only allowed complementation, but also acted as a selectable marker. Removal of the kanamycin resistance gene, which remained necessary for plasmid manipulations in *Escherichia coli*, was accomplished by two different methods: restriction enzyme digestion followed by re-ligation before BCG transformation, or by Cre–*loxP in vitro* recombination mediated by the bacteriophage P1 Cre Recombinase. Stability of the plasmid was evaluated during *in vitro* and *in vivo* growth of the recombinant BCG in comparison to selection by antibiotic resistance. The new system was highly stable even during *in vivo* growth, as the selective pressure is maintained, whereas the conventional vector was unstable in the absence of selective pressure. This new system will now allow the construction of potential recombinante vaccine strains using stable multicopy plasmid vectors without the inclusion of antibiotic resistance markers.

*Corresponding author. Tel.: +55 53 32757350.
E-mail address: odirad@terra.com.br (O.A. Dellagostin).

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Introduction

The live attenuated *Mycobacterium bovis* strain Bacille Calmette-Guerin (BCG) is widely used as a vaccine against tuberculosis. It also has many properties that make it one of the most attractive live vectors for the development of recombinant vaccines against other diseases, for example: it is unaffected by maternal antibodies and therefore it can be given at any time after birth; it is usually given as a single dose eliciting both humoral and cell-mediated immune responses; it is stable and safe; it can be administrated orally; and it is inexpensive to produce when compared to other live vaccines.[1,2] Development of expression vectors for mycobacteria has allowed the construction of recombinant BCG (rBCG) that express a variety of foreign antigens that, in some instances, induced protective immune responses in animal models.[3–6] Recombinant BCG has also been used to express *Mycobacterium tuberculosis* proteins for developing an improved vaccine against tuberculosis.[7] Expression of the *M. tuberculosis* 30 kDa major secretory protein in BCG resulted in greater protective immunity than wild type BCG against human[8] and bovine tuberculosis.[9]

A large number of *Escherichia coli*-mycobacterium shuttle vectors have been developed for the transfer of foreign genes into BCG. These are maintained in mycobacteria either episomally or by integrating into the mycobacterial genome. Most episomal plasmids have been developed by combining a region of the mycobacterial replicon of the *Mycobacterium fortuitum* pAL5000 plasmid[10] with an *E. coli* cloning vector and a kanamycin resistance gene.[11] These shuttle vectors replicate in mycobacteria at about five copies per genome.[1] However, expression systems for heterologous genes in BCG that use episomal vectors are frequently unstable in the absence of the selection.[12–16] Integrative vectors, derived from temperate mycobacteriophages, such as L5[17] or Ms6,[16] have also been developed. These integration-proficient vectors encode integrase functions that allow a recombination event between the phage *attP* and the bacterial homologous *attB* sites. These vectors are more stable, but are integrated into the mycobacterial genome as a single copy.[17,18] The lack of stability of episomal vectors and the low copy number of integrative vector can compromise the expression of heterologous antigen in BCG and influence the immune response to foreign antigens.

Expression systems currently used in BCG rely on antibiotic resistance genes as selectable markers.[11] However, these markers do not provide selection for the vaccine *in vivo*, nor are they desirable in a practical recombinant BCG based vaccine. Therefore, the development of more stable plasmid vectors for heterologous antigen expression in BCG that do not carry antibiotic resistance marker is necessary. Superinfection immunity to mycobacteriophages[19] and resistance to mercury[20] have been used as selectable markers in mycobacteria, but these still do not provide *in vivo* selective pressure.

In this report, we describe the construction of a BCG expression system using auxotrophic complementation as a selectable marker. A BCG Δ*leuD* was obtained by gene knock-out, and a plasmid encoded *leuD* gene used as a selectable marker. The kanamycin resistance gene, necessary for selection in *E. coli* during plasmid construction, was removed prior to BCG transformation by two different strategies: bacteriophage P1 Cre–*loxP* site-specific recombination or restriction enzyme digestion followed by religation. The new selection system resulted in remarkably improved stability of the vector as the selective pressure is maintained when the rBCG is inside macrophage cells.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli strain TOP10 (Invitrogen) was grown in Luria–Bertani medium at 37 °C with the addition of the appropriate antibiotic (kanamycin 50 μg/ml or hygromycin 200 μg/ml). *M. bovis* BCG Pasteur, *Mycobacterium smegmatis* mc^{2}155 and BCG Δ*leuD* were grown in Middlebrook 7H9 broth (Difco) supplemented with 10% of oleic acid, albumin, dextrose complex (OADC—Difco), 0.2% glycerol and 0.05% Tween 80 (Sigma), or 7H10 and 7H11 agar (Difco) containing 10% OADC and 0.2% glycerol. When necessary, the auxotrophic strains were grown in media supplemented with 100 μg/ml L-leucine (Sigma), 25 μg/ml kanamycin (Sigma) or 50 μg/ml hygromycin B (Invitrogen) and 40 μg/ml X-Gal (Sigma).

Experimental animals

BALB/c mice (4–6 weeks old) were obtained from the Central Animal Facility of the Federal University of Pelotas. The experimental animals were housed at the animal facility of the Biotechnology Centre of the Federal University of Pelotas (UFPel). The animals were maintained in accordance with the guidelines of the UFPel Ethics Committee in Animal Experimentation throughout the experimental period.

Construction of *leuD* gene knock-outs in *M. bovis* BCG Pasteur strain 1173P2 and *M. smegmatis* mc^{2}155

Unmarked BCG Δ*leuD* and *M. smegmatis* Δ*leuD* were constructed as described by Parish and Stoker.[21] The homologous chromosomal regions were PCR amplified from regions upstream (BCG-HR1-F 5′-CTACCTGCAGCAACGTGCGGGCCTAACACACGGATA and BCG-HR1-R 5′-CATCAAGCTTGGATCGAGGCCTTTGAGGGT) and downstream (BCG-HR2-F 5′-CTCAAACGTTGCTGATCCCCTAGCTGTTCT and BCG-HR2-R 5′-CATCGGATCCTAGCCACACCTCAACCCAC) of the BCG *leuD* and from the regions upstream (Ms-HR1-F 5′-GCGACTGCAGATCGGAAAGGCAGCACCT and Ms-HR1-R 5′-ATCCAAGCTTCCACCTAGTCGGTTCTGGAG) and downstream (Ms-HR2-F 5′-ATCGAAGCTTGCTTCTCCTTCGTGGGTTTT and Ms-HR2-R 5′-ATTAGGATCCCTCAAGCCCTTCAAGACCAT) of the *M. smegmatis leuD*. These were cloned into p2-NIL using *Pst*I/*Hin*dIII and *Hin*dIII/*Bam*HI and the resultant plasmid ligated to the pGOAL-19 *Pac*I fragment.[22]

To transform the knock-out plasmids into mycobacteria, cultures in logarithmic growth were harvested by centrifugation. The pellet was washed in ×1, ×0.5 and ×0.2 volumes of 10% glycerol (37 °C for BCG and 4 °C for *M. smegmatis*) and the cells finally resuspended in ×0.01

volume. A 200 μl aliquot of competent cells were transformed by electroporation (25 μF, 1000 Ω, 2.5 V for BCG and 25 μF, 600 Ω, 2.5 V for *M. smegmatis*) with approximately 1 μg UV irradiated (100 mJ/cm^2) plasmid. BCG cells were recovered in 10 ml 7H9 for 12–24 h and plated on 7H11 with kanamycin and X-Gal while *M. smegmatis* cells were recovered for 4 h in Nutrient Broth II and plated on Nutrient Agar II with kanamycin and X-Gal. Colonies with β-galactosidase activity were sub-cultured onto plates containing X-Gal and 10% sucrose. White colonies were selected and their genotype determined by PCR using BCG-HR1-F and BCG-HR2-R, and Ms-HR1-F and Ms-HR2-R; and by Southern blot using probes containing the homologous regions, the *leuD* gene and pGOAL-19. The auxotrophy of the Δ*leuD* isolates of both BCG and *M. smegmatis* was tested by culturing in 7H9 and on 7H10 media, with and without leucine.

Auxotrophic complementation vector construction

A schematic representation of the steps involved in the design of the vectors is provided in Figure 1. The *leuD* gene coding sequence was PCR amplified from *M. bovis* BCG P3 DNA with primers *leuD*I (5′-AATCTAGAACAGCTAGGGGATC), and *leuD*II (5′-TCCTGCAGTTCTACGCCTCA). The amplified fragment was digested with *Xba*I and *Pst*I and cloned into pUS977[13] to generate pUP400. The Hyg^R cassette from pGOAL19[22] was obtained by *Kpn*I digestion and ligated into *Kpn*I digested pUP400 to give pUP401.

Two methods were used to enable the kanamycin resistance gene to be removed from the vector prior to transformation into BCG. To remove the resistance gene by digestion and re-ligation, an adaptor that carries the *Hin*dIII site with *Pac*I compatible ends was ligated into the *Pac*I site of pUP401. Adap-F (5′-GATATCAAGCTTAAGACGCGTTAA) and Adap-R (5′-TAACGCGTCTTAAGCTTGATATCTA) were hybridized by boiling for 1 min and incubated for 3 h at room temperature. Hybridized oligonucleotides (500 ng) were ligated to *Pac*I digested pUP401 (200 ng). The ligation mix was heated at 70 °C for 10 min, and immediately electrophoresed on 1% agarose gel. The ligation product was excised and eluted from the gel using GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Biosciencies). Hybridization buffer (10 mM Tris–HCl, pH 8.5; 100 mM NaCl; 1 mM EDTA) was added to the eluted DNA and the mix heated at 80 °C for 5 min, kept at room temperature for 3 h and used to transform *E. coli* TOP10 cells. Recombinant clones were identified by digestion with *Hin*dIII and by DNA sequencing (MegaBACE—Amersham Biosciences). The resultant plasmid was named pUP402.

To enable the kanamycin resistance gene to be removed by site-specific recombination, oligonucleotides containing the Cre–*loxP* site with either terminal *Hin*dIII sites or terminal *Pac*I sites were synthesized. The Cre–*loxP*–*Hin*dIII F (5′-TATAACTTCGTATAATGTATGCTATACGAAGTTAT) and Cre–*loxP*–*Hin*dIII R (5′-ATAACTTCGTATAGCATACATTATACGAAGTTA-TA) oligonucleotides were hybridized and ligated into *Hin*dIII digested pUP401 vector to give pUP401-lox–*Hin*dIII.

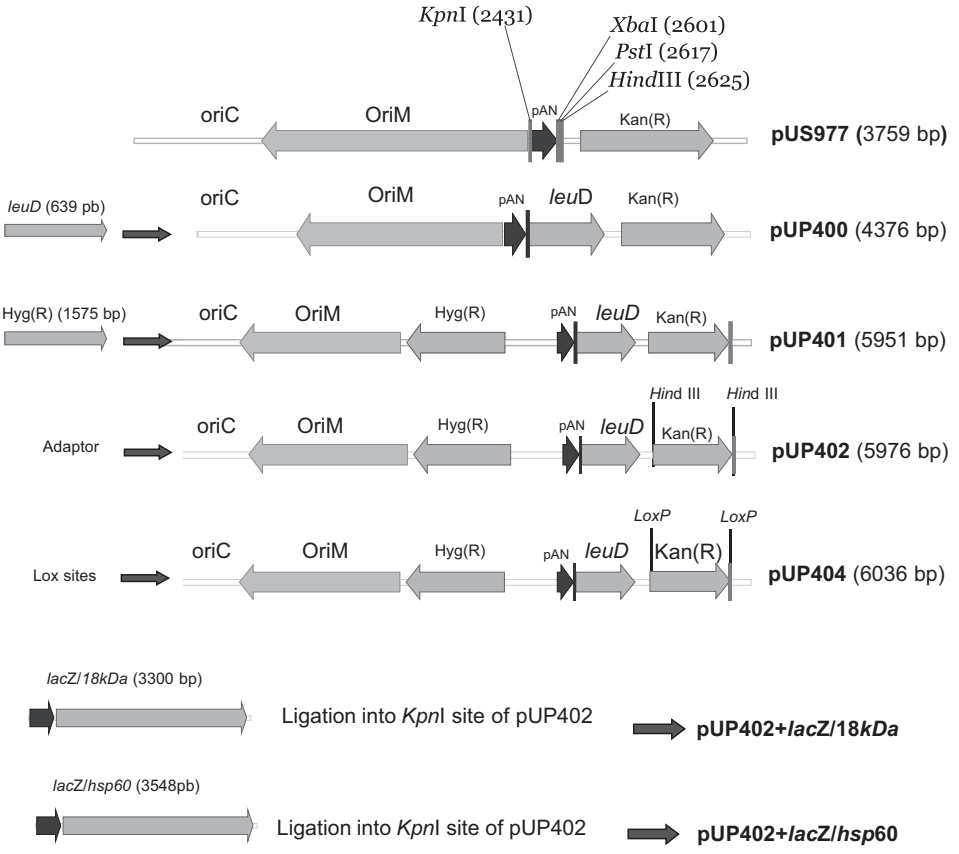

Figure 1 Schematic representation of the strategy employed in this study for the construction of pUP vector series. The *lac*Z gene replaced the hygromycin resistance gene in pUP402+*lac*Z/18 kDa and pUP402+*lac*Z/*hsp*60.

Cre–*loxP*–*Pac*I F (5′-TAAATACTTCGTATAATGTATGCTATACGAAGTTATAT) and Cre–*loxP*–*Pac*I R (5′-ATATAACTTCGTATAGCATACATTATACGAAGTATTTA) oligonucleotides were hybridized and ligated into pUP401-lox–*Hin*dIII digested with *Pac*I as described for the *Hin*dIII–*Pac*I adaptor. The presence and orientation of the *loxP* sites were determined by restriction digestion and sequencing. The resulting vector was named pUP404.

To assess functional stability of the *leuD* complementation system *in vivo*, a *lac*Z gene in fusion with *hsp60* or 18 kDa promoters was used. The *lac*Z–*hsp*60 and *lac*Z–18 kDa cassettes were PCR amplified from pUS985 and pUS993 vectors, respectively,[13] using Hsp60 F (5′-TCGGTACCCCGACCACAACGACG), and 18 kDa F (5′-TCGGTACCGCAGCGACGGCACCGG) with the *lac*Z R primer (5′-TCGGTACCTTATTTTTGACACCAGAC). The fragments were ligated into the *Kpn*I site of the pUP402 vector, replacing the hyg^R gene. The resulting vectors were named pUP402+*lacZ*/*hsp*60 and pUP402+*lacZ*/18 kDa.

Removal of the kanamycin resistance gene

To remove the kanamycin resistance gene by digestion/re-ligation, pUP402 (1 μg) was digested with *Hin*dIII and re-ligated with T4 DNA ligase. To remove the kanamycin resistance gene by *in vitro* site-specific recombination, pUP404 (1 μg) was incubated for 1 h with 3 U Cre Recombinase (Biolabs). *M. bovis* BCG Δ*leuD* and *M. smegmatis* mc^2155 Δ*leuD* electrocompetent cells were transformed with the product of the ligation reaction (pUP402 Δkan^R) or recombination reaction (pUP404 Δkan^R). Transformants were plated on 7H10 with and without kanamycin and the colony counts compared to evaluate the efficiency of kanamycin resistance gene removal. These experiments were carried out in triplicate.

In vitro stability analysis

Cultures of *M. smegmatis* mc^2155 Δ*leuD* transformed with pUP402 Δkan^R vector; BCG Δ*leuD* and BCG Pasteur transformed with pUP402, pUP402 Δkan^R, pUP402+*lacZ*/*hsp*60 or pUP402+*lacZ*/18 kDa were grown in 7H9 broth with and without selection (L-leucine for Δ*leuD* strains and kanamycin for rBCG Pasteur). Transformed BCG Δ*leuD* and *M. smegmatis* mc^2155 Δ*leuD* were sub-cultured 7 times and 20 times, respectively, by transferring 62.5 μl of the stationary phase culture to 2 ml of fresh medium every 7 days for BCG and every 2 days for *M. smegmatis* mc^2155. Every three or four sub-cultures, the number of colonies on selective and non-selective 7H10 plates containing X-Gal were compared. Every four sub-cultures total DNA was extracted, transformed into *E. coli* TOP10 cells and plated on LB agar containing hygromycin for pUP402 Δkan^R, or kanamycin for pUP402+*lacZ*/*hsp*60, pUP402+*lacZ*/18 kDa and pUP402. The restriction digestion banding pattern of plasmids from five individual colonies from each transformation were determined in order to evaluate the plasmid's structural stability.

β-Galactosidase assay

β-Galactosidase activity was assayed in BCG Δ*leuD* and BCG Pasteur as previously described.[23] Briefly, duplicate 0.2 ml volume of test cultures (OD_{600} 0.8–1.4) were added to 0.3 ml Z buffer (60 mM NaH_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mecaptoethanol) and 0.5 ml of glass beads 0.1 mm (Biospec Products). The cells were lysed in a Hybaid Ribolyser (Thermo Hybaid) for 40 s at power 5, centrifuged and the supernatant incubated at 28 °C for 5 min. A volume of 200 μl ONPG 4 mg/ml (Sigma) was added and the reaction incubated at 28 °C. When sufficient yellow colour developed, the reaction was stopped by adding 200 μl of 1 M Na_2CO_3 and the incubation time recorded. The OD_{420} of each reaction mix was measured and the β-galactosidase activity was calculated.[23]

In vivo stability analysis of the rBCG

Four to 6-week-old BALB/c mice were used to evaluate the *in vivo* stability of the rBCG. In the first experiment 48 animals were randomly allocated into six groups, eight animals per group, and inoculated intraperitoneally with approximately 5×10^5 cfu of rBCG in 100 μl of sterile PBS-T. Group 1 was inoculated with rBCG Δ*leuD* (pUP402+*lacZ*/*hsp*60), group 2 with rBCG Δ*leuD* (pUP402+*lacZ*/18 kDa), group 3 with rBCG Pasteur (pUP402+*lacZ*/*hsp*60), group 4 with rBCG Pasteur (pUP402+*lacZ*/18 kDa), group 5 with rBCG Δ*leuD* (pUP402), and group 6 with rBCG Pasteur (pUP402). At weeks 2, 5, 8, 12, 16 and 30 after inoculation the spleen from one mouse per group was removed, homogenized, serially diluted in 7H9, and plated onto 7H10 with X-Gal and with or without selection (L-leucine for rBCG Δ*leuD*, and kanamycin for rBCG Pasteur). A second experiment was carried out with the same number of animals per group, however spleen from two mice was removed at each time point. The number of resultant colonies was compared and the percentage of blue colonies determined.

Results

M. bovis BCG Δ*leuD* and *M. smegmatis* Δ*leuD* construction

Unmarked *M. bovis* BCG Δ*leuD* and *M. smegmatis* Δ*leuD* were successfully constructed by homologous recombination using the strategy of Parish and Stoker.[22] Genotypes were confirmed by PCR and Southern blots, both giving bands that agreed with the predicted knock-out genotype. The resultant Δ*leuD* strains were not able to grow on 7H10 or in 7H9 without the addition of leucine.

Evaluation of *leuD* as a selectable marker

In order to evaluate the usefulness of auxotrophic complementation as a selectable marker, the *leuD* coding sequence was amplified by PCR and cloned into pUS977 under the control of P_{AN} promoter. The resulting vector, named pUP400 (Figure 1), was used to transform BCG Δ*leuD* and *M. smegmatis* mc^2155 Δ*leuD*. Transformation rates were similar using either auxotrophic complementation or kanamycin resistance selection methods, approximately 6×10^2 cfu/μg DNA for *M. bovis* BCG Δ*leuD*, and 3.6×10^3 cfu/μg of DNA for *M. smegmatis* mc^2155 Δ*leuD*. Thus, the *leuD*

complementation system and kanamycin resistance had equivalent selective capacities.

Removal of the kanamycin resistance gene

Two different approaches were evaluated to remove the kanamycin resistance gene from the plasmid prior to transformation into mycobacteria: restriction enzyme digestion followed by re-ligation of the vector; and bacteriophage P1 Cre–*loxP* site-specific recombination system. An oligonucleotide containing a *Hin*dIII site was cloned downstream of the kanamycin gene, resulting in two *Hin*dIII sites, one either side of the kanamycin gene. Digestion and re-ligation of the resulting vector, named pUP402, was carried out just before transformation of both BCG Δ*leuD* or *M. smegmatis* mc^{2}155 Δ*leuD*.

The second approach consisted of cloning oligonucleotides containing Cre–*loxP* sites flanking the kanamycin resistance gene. *In vitro* treatment of pUP404 with Cre Recombinase resulted in excision of the kanamycin resistance gene. The DNA band corresponding to the kanamycin resistance gene excised from the plasmid after treatment with Cre Recombinase could be observed by agarose gel electrophoresis (data not shown).

Transformation efficiency with re-ligated DNA or with Cre Recombinase treated DNA was approximately 1 log lower than with intact plasmid. The efficiency of removal of the kanamycin gene was evaluated by replica plating 100 individual transformants on plates with and without kanamycin. For pUP402 digested with *Hin*dIII and re-ligated, 95% of the colonies failed to grow on kanamycin, confirming that the kanamycin resistance gene had been efficiently removed. The same vector when used to transform *M. smegmatis* mc^{2}155 Δ*leuD* revealed a removal efficiency of 85%. Removal of the kanamycin resistance gene from pUP404 by site-specific recombination was less efficient. Only 41% of *M. bovis* BCG Δ*leuD* transformed with Cre Recombinase treated vector lost the kanamycin resistance gene, whereas in *M. smegmatis* mc^{2}155 Δ*leuD* this number was 31%.

In vitro stability analysis of recombinant mycobacteria

To evaluate the *in vitro* persistence of the plasmids harbouring the auxotrophic complementation *leuD* gene, sub-cultures on media with and without selection were carried out. All of the *M. smegmatis* mc^{2}155 Δ*leuD* strain cultured on selective medium (without leucine supplementation) maintained the vector for over 20 sub-cultures (approximately 190 generations). However, only 20% of the colonies still carried the vector after only four sub-culturing passages (approximately 40 generations) without selective pressure (with leucine). This decreased to only 4% after 20 passages (Figure 2).

In order to evaluate the functional stability of genes carried by the complementation system, *lacZ* was used as a reporter gene under two different mycobacterial promoters: the *hsp60* promoter (pUP402+*lacZ*/*hsp60*) and the 18 kDa promoter from *Mycobacterium leprae* (pUP402+*lacZ*/18 kDa). Both vectors were used to transform *M. bovis* BCG

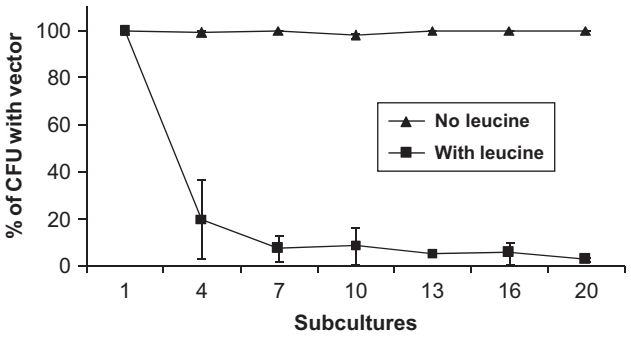

Figure 2 *In vitro* stability of pUP402 in *M. smegmatis* mc^{2}155 Δ*leuD* grown for successive passages on selective (no leucine) or non-selective (with the addition of leucine) media. The percentage represents the number of CFU that still maintained the vector (growth on selective medium) in relation to the total number of CFU. Twenty sub-cultures represent approximately 190 generations. Average of the four individual clones is shown.

Δ*leuD* and *M. bovis* BCG Pasteur. When bacteria were grown under selective pressure, the level of expression of *lacZ* determined by β-galactosidase enzyme activity assay, remained stable with successive passages, however in the presence of leucine for rBCG Δ*leuD* or absence of kanamycin for rBCG Pasteur, the level of expression dropped. In the case of BCG Pasteur transformed with pUP402+*lacZ*/*hsp60* and grown under non-selective condition, after six passages β-galactosidase activity was completely abolished. To ascertain that this effect was not due to mutations in the expression cassette, cultures grown without selective pressure were plated out on selective and non-selective media containing X-Gal. The small number of colonies obtained on selective medium was blue, whereas on non-selective medium a large number of white colonies were obtained, demonstrating that the drop in *lacZ* expression was as a result of loss of the vector.

Structural stability of the vectors was evaluated by plasmid rescuing and analysis of restriction digest patterns. Every four sub-cultures of *M. smegmatis* mc^{2}155 Δ*leuD* transformed with pUP202 Δkan^R or rBCG Δ*leuD* (pUP402+*lacZ*/*hsp*60, pUP402+*lacZ*/18 kDa and pUP402) and rBCG Pasteur (pUP402+*lacZ*/*hsp*60, pUP402+*lacZ*/18 kDa and pUP402) total DNA was extracted and used to transform *E. coli*. Hygromycin selection was used throughout the plasmid rescuing experiments to distinguish between untransformed, plasmid-free bacteria as some vectors had the kanamycin resistance gene removed. Plasmid DNA was extracted from individual colonies and restriction digested. The resulting restriction patterns were compared with those of the vectors originally used to transform the mycobacterial hosts. Gross modifications in vector structure were not observed (data not shown).

In vivo stability analysis of the rBCG

Stability of *M. bovis* BCG Δ*leuD* and *M. bovis* BCG Pasteur transformed with plasmids containing the *leuD* gene as selectable marker was evaluated in mice. Three different

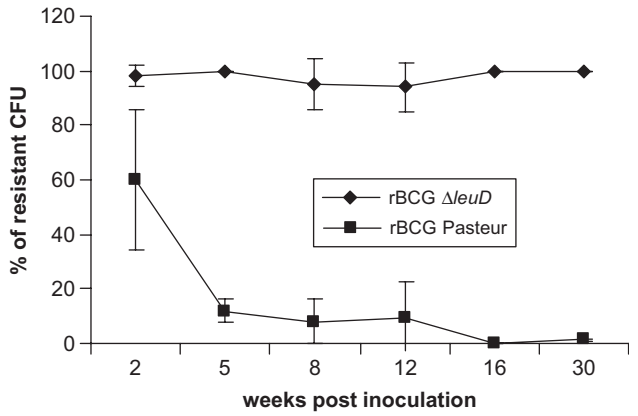

Figure 3 *In vivo* stability of rBCG transformed with vectors containing auxotrophic complementation. Mice were inoculated with BCG Δ*leuD* transformed with pUP402, pUP402+*lacZ*/*hsp*60 or pUP402+*lacZ*/18 kDa and BCG Pasteur transformed with the same vectors. Bacteria were recovered from spleen of three animals killed at 2, 5, 8, 12, 16 and 30 weeks post-inoculation and plated on selective and non-selective medium. Ratios of resistant (rBCG) versus total BCG colonies were calculated for each strain. Average of the three BCG Δ*leuD* and three BCG Pasteur strains is shown.

plasmids were used to transform both BCG strains. Initial selection was carried out on medium without leucine for BCG Δ*leuD* or containing kanamycin for BCG Pasteur. Approximately 5×10^5 cfu of each strain were inoculated into mice. Auxotrophic complementation vectors used to transform BCG Δ*leuD* showed 90–100% of stability *in vivo* during the 30 weeks of the experiment (Figure 3). There was no significant difference among vectors in terms of stability. Conversely, the same vectors used to transform BCG Pasteur were lost during *in vivo* growth. Five weeks post-inoculation less than 20% of the bacterial cells still had the plasmid. This number fell to zero at 16 weeks post-inoculation (Figure 3). A very high level of functional stability was also observed as all the recovered BCG Δ*leuD* transformed with vectors carrying *lac*Z gave rise to blue colonies when plated onto medium containing X-Gal.

Discussion

A highly stable plasmid vector for cloning and expression of foreign genes in BCG has been developed in this study. This system is based on the use of a *M. bovis* BCG strain auxotrophic for the leucine amino acid obtained by knocking out the *leuD* gene, and complementation with *leuD* inserted into the plasmid vector, which acts as a selectable marker. This approach has two main advantages: it provides active selection *in vivo*, unlike antibiotic resistance markers, and it abolishes the need for using an antibiotic resistance gene as a vector component.

The majority of currently used mycobacterial vectors carry the *Tn*5 or *Tn*903-derived kanamycin resistance.[11,18] Mercury resistance and superinfection immunity to mycobacteriophage L5 have also been used as selectable markers in mycobacteria, but with limited success.[19,20,24] None of the selectable markers used so far are active *in vivo*. For many applications this is not an issue, but for expression of foreign antigens with the aim of developing BCG into a multivalent recombinant vaccine, or an improved vaccine against tuberculosis, *in vivo* vector stability is critical. Loss of the plasmid vector has been implicated in failure of recombinant BCG vaccine candidates.[16,25]

With the aim of developing an efficient mechanism of removal of the kanamycin resistance gene, the Cre–*loxP* site-specific recombination system[26] was employed. A simpler approach was also developed by inserting an oligonucleotide containing a *Hin*dIII site downstream of the kanamycin resistance gene, resulting in two *Hin*dIII sites flanking the gene. Digestion with *Hin*dIII followed by religation using a concentration of DNA that favoured intra molecular ligation resulted in removal of the kanamycin gene with a significantly higher efficiency than that obtained with the Cre–*loxP* system. In the end, both systems were functional and either could be used.

The use of auxotrophic complementation as selectable marker for expression of foreign genes in BCG described in this report provides stability previously described only for integrative vectors,[16,27] with the advantages of a multicopy vector, namely higher levels of foreign antigen expression. In addition, since no antibiotic resistance gene is required, the system is applicable to the development of recombinant live vaccines. Evaluation of the immune response obtained with the use of this new selection system is currently been performed with several recombinant antigens. It is expected that it will be able to elicit a strong and long-lasting immune response against these antigens, contributing to the development of BCG into a multivalent vaccine.

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References

1. Stover CK, Delacruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, et al. New use of BCG for recombinant vaccines. *Nature* 1991;**351**:456–60.
2. Bloom BR, Fine PEM. The BCG experience: implications for future vaccines against tuberculosis. In: Bloom BR, editor. *Tuberculosis: pathogenicity, protection and control*. Washington, DC: American Society for Microbiology; 1994. p. 531–57.
3. Abdelhak S, Louzir H, Timm J, Blel L, Benlasfar Z, Lagranderie M, et al. Recombinant BCG expressing the *Leishmania* surface-antigen Gp63 induces protective immunity against *Leishmania major* infection in BALB/c mice. *Microbiology* 1995;**141**:1585–92.

4. Bastos RG, Dellagostin OA, Barletta RG, Doster AR, Nelson E, Zuckermann F, et al. Immune response of pigs inoculated with *Mycobacterium bovis* BCG expressing a truncated form of GP5 and M protein of porcine reproductive and respiratory syndrome virus. *Vaccine* 2004;**22**:467–74.
5. Matsumoto S, Yukitake H, Kanbara H, Yamada T. 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* 1998;**188**:845–54.
6. Ohara N, Yamada T. Recombinant BCG vaccines. *Vaccine* 2001;**19**:4089–98.
7. Horwitz MA. Recombinant BCG expressing *Mycobacterium tuberculosis* major extracellular proteins. *Microbes Infect* 2005;**7**:947–54.
8. Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. 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 USA* 2000;**97**:13853–8.
9. Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. A novel live recombinant mycobacterial vaccine against bovine tuberculosis more potent than BCG. *Vaccine* 2006;**24**:1593–600.
10. Labidi A, David HL, Roullanddussoix D. Cloning and expression of mycobacterial plasmid DNA in *Escherichia coli*. *FEMS Microbiol Lett* 1985;**30**:221–5.
11. Snapper SB, Lugosi L, Jekkel A, Melton RE, Kieser T, Bloom BR, et al. Lysogeny and transformation in mycobacteria-stable expression of foreign genes. *Proc Natl Acad Sci USA* 1988;**85**: 6987–91.
12. Lagranderie M, Murray A, Gicquel B, Leclerc C, Gheorghiu M. Oral immunization with recombinant BCG induces cellular and humoral immune-responses against the foreign antigen. *Vaccine* 1993;**11**:1283–90.
13. Medeiros MA, Dellagostin OA, Armoa GRG, Degrave WM, 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.
14. Murray A, Winter N, Lagranderie M, Hill DF, Rauzier J, Timm J, et al. 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* 1992;**6**:3331–42.
15. Nascimento IP, Dias WO, Mazzantini RP, Miyaji EN, Gamberini M, Quintilio W, et al. Recombinant *Mycobacterium bovis* BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live *Bordetella pertussis* in mice. *Infect Immun* 2000;**68**:4877–83.
16. Mederle I, Bourguin I, Ensergueix D, Badell E, Moniz-Peireira J, Gicquel B, et al. Plasmidic versus insertional cloning of heterologous genes in *Mycobacterium bovis* BCG: impact on in vivo antigen persistence and immune responses. *Infect Immun* 2002;**70**:303–14.
17. Lee MH, Pascopella L, Jacobs WR, Hatfull GF. Site-specific integration of mycobacteriophage-L5-integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and Bacille Calmette-Guerin. *Proc Natl Acad Sci USA* 1991;**88**:3111–5.
18. Dellagostin OA, Wall S, Norman E, Oshaughnessy T, Dale JW, McFadden J. Construction and use of integrative vectors to express foreign genes in mycobacteria. *Mol Microbiol* 1993; **10**:983–93.
19. Donnellywu MK, Jacobs WR, Hatfull GF. Superinfection immunity of mycobacteriophage-L5-applications for genetic transformation of mycobacteria. *Mol Microbiol* 1993;**7**:407–17.
20. Baulard A, Escuyer V, Haddad N, Kremer L, Locht C, Berche P. Mercury resistance as a selective marker for recombinant mycobacteria. *Microbiology* 1995;**141**:1045–50.
21. Parish T, Stoker NG. *Mycobacteria protocols*. NJ: Humana Press Inc.; 1998.
22. Parish T, Stoker NG. Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis tlyA plcABC* mutant by gene replacement. *Microbiology* 2000;**146**: 1969–75.
23. Dellagostin OA, Esposito G, Eales LJ, Dale JW, McFadden J. Activity of mycobacterial promoters during intracellular and extracellular growth. *Microbiology* 1995;**141**:1785–92.
24. Reyrat JM, Pelicic V, Gicquel B, Rappuoli R. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infect Immun* 1998;**66**:4011–7.
25. 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.
26. Sternberg N, Hamilton D. Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol* 1981;**150**:467–86.
27. Vultos TD, Mederle I, Abadie V, Pimentel M, Moniz-Pereira J, Gicquel B, et al. Modification of the mycobacteriophage Ms6 attP core allows the integration of multiple vectors into different tRNAala T-loops in slow- and fast-growing mycobacteria. *BMC Mol Biol* 2006;**7**:47.