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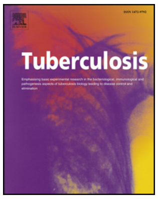

Molecular Aspects

A standardized BioBrick toolbox for the assembly of sequences in mycobacteria

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ABSTRACT

For more than 25 years, recombinant *Mycobacterium bovis* BCG has been genetically engineered for use as a vehicle for antigen expression and immunomodulation, typically through introducing or deleting a gene from BCG genome. However, BCG transformation efficacy is still unpredictable, and cloning and expression of sequences from mycobacteria is difficult to predict due to the lack of standardization. To overcome such limitations, we have employed the BioBrick format to construct a toolbox of several mycobacterial parts, including coding sequences, reporter genes, selective markers, promoters, and other regulatory sequences. Additionally, we have developed and characterized BioBrick-compatible episomal vectors that are able to replicate in *M. bovis* BCG to enable expression of heterologous antigens. The availability of a BCG Biobrick toolbox will enable any coding sequence to be optimally expressed in BCG. We believe that this mycobacterial toolbox represents a standardized and useful kit to enhance the efficacy and use of recombinant BCG.

1. Introduction

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

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

Mycobacterium bovis Bacillus Calmette–Guerin (BCG) is an attenuated vaccine widely used against tuberculosis (TB) that also offers the potential to be used as a live vaccine vector [11]. BCG is currently the world's most widely used vaccine and has been given to more than three billion people. It has many advantages as a vaccine: it can be given at birth; it replicates in antigen presenting cells such as macrophages and dendritic cells, so is very effective at presenting antigens to the host immune system and thereby confers long-lasting protection with high levels of both Th1 and Th2 cellular immunity; the mycobacterial cell wall is one of the strongest immunological adjuvants (Freunds Complete Adjuvant is a killed preparation of Mtb in oil); it is associated with a very low rate of complications; it is stable and so does not require a cold chain for implementation [11].

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Since their initial development in the 1990's, rBCG vaccines have been developed that express homologous and heterologous antigens from a wide range of pathogens [11–13]. Delivery of the heterologous (or native) gene(s) has usually been via a plasmid or integrative vector. Expression of the foreign genes is achieved by placing it under the control of a range of mycobacterial promoters [13]. Delivery of the foreign antigen to the BCG surface or their secretion for extracellular medium have been engineered in some systems [13].

Despite all these approaches, the need to establish uniform mycobacterial cloning platforms has only recently begun to be addressed, in a study developed by Arnold et al. [14]. The authors developed a series of mycobacterial vectors for protein production using the fragment exchange (FX) cloning strategy and validated their functionality in the model *M. smegmatis* [14]. It is well known that promoter strength, cellular location of foreign antigens (e.g., in mycobacterial cytoplasm, associated to cell wall or in a secreted form), and the presence or absence of antibiotic resistance genes are determinant factors for the stability and efficacy of rBCG strains [15]. These factors are determined by different DNA sequences, which are frequently shared in a variety of projects with rBCG [13]. Standardization of these sequences would simplify assembly and allow reproducibility in construction of rBCG strains.

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

2. Material and methods

2.1. Bacterial strains and growth conditions

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

2.2. Reagents and DNA manipulation

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

2.3. Construction of standard basic parts

The promoters P_{pAN}, P_{18kDa}, and P_{hsp60} were amplified, respectively, from the following plasmids previously constructed: pUS977, pUS2000, and pUS973 [16,17] (Table 1). The promoters P_{ag85B}, P_{sag85B}, P_{hspX} and its truncated sequence (P_{hspXT}) were obtained from genomic DNA of *M. bovis* BCG Pasteur. The promoters PL5X, PL5Y, and PL5Z were generated by error-prone PCR of the PL5 promoter in a previous work [18]. The *leuD* sequence was obtained from pUP410 [19]. The *ag85b* coding sequence and the mycobacterial origin of replication were commercially synthesized by GenOne (Rio de Janeiro, Brazil) to remove BioBrick restriction sites in their sequences and then amplified by PCR. All fragments were cut with *Eco*RI and *Pst*I, ligated into pSB1C3 (BioBrick submission vector), and confirmed by sequencing. A BioBrick replicative vector for expression in BCG, named pUP500, was commercially synthesized by GenOne (Rio de Janeiro, Brazil) with the *E. coli* origin of replication, a resistance gene to kanamycin flanked by *Hind*III restriction sites and the BioBrick multiple cloning site containing the prefix and suffix sequences. Moreover, pUP500 has a *Nhe*I restriction site where the mycobacterial origin of replication was inserted. Briefly, the mycobacterial origin of replication was amplified by PCR, digested with *Xba*I and *Spe*I, and ligated into pUP500 previously digested with *Nhe*I. Afterwards, *E. coli* DH5α competent cells were electroporated and plated on selective medium. Recombinant clones were confirmed by PCR, digestion, and sequencing. To access the functionality of pUP500 for replication in BCG, competent cells of BCG Pasteur were electroporated and plated on selective medium.

2.4. Assembly of BioBrick vectors for expression in rBCG

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

2.5. BCG transformation and analysis of expression

Electrocompetent cells of *M. bovis* BCG Pasteur were transformed with the plasmids pUP500/PpAN:*egfp*, pUP500/P18 kDa:*egfp*, pUP500/Phsp60:*egfp*, pUP500/Pag85B:*egfp*, pUP500/Psag85B:*egfp*, pUP500/PhspX:*egfp*,and pUP500/PhspXT:*egfp*, and recombinant strains were selected in 7H10 medium with kanamycin. Recombinant BCG strains were grown for 5 days in selective 7H9 medium and visualized with a fluorescence microscope at 200 × magnification.

2.6. Macrophage infection

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

2.7. Characterization of eGFP expression in rBCG strains

An Attune® Acoustic Focusing Cytometer, Blue/Violet (Applied Biosystems, California, United States) was used to determine the single-cell fluorescence of gated bacterial cells. Others events were eliminated from analysis by scatter plots of FSC × SSC and negative/positive fluorescence of eGFP (with 488 nm laser and 530/30 nm emission filter). The acquisition rate was 200 events s^{-1} totaling of 20,000 bacterial cells per sample were collected. Expression of eGFP was evaluated in terms of percentage of bacterial cells with positive eGFP (Bacterial cells with high fluorescence/Bacterial cells with High + Low fluorescence) and median intensity of green fluorescence among positive cells.

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

BioBrick part	Primers (5′-3′)	Reference of source
$\mathbf{P_{paN}}$	F: CCGGAATTCGCGGCCGCTTCTAGACCAAGGCCGAAGAGCCC R: TGCACTGCAGCGGCCGCTACTAGTATCCCTTGACAACGTCAT	**pUS977** [16]
$\mathbf{P_{hsp60}}$	F: CCGGAATTCGCGGCCGCTTCTAGAGACCACAACGACGCGCCC R: TGCACTGCAGCGGCCGCTACTAGTCGTCTTGGCCATTGCGAA	**pUS973** [16]
$\mathbf{P_{18kDa}}$	F: CGGAATTCGCGGCCGCTTCTAGAGGTACCGCAGCGACG R: TGCACTGCAGCGGCCGCTACTAGTCATCAGCATGTGTGGTC	**pUS2000** [19]
$\mathbf{P_{ag85B}}$	F: CCGGAATTCGCGGCCGCTTCTAGACGCTATGTAGCTCCA R: TGCACTGCAGCGGCCGCTACTAGTACCTGTGCCCCTTTG	**gDNA from *M. bovis* BCG Pasteur**
$\mathbf{P_{sag85B}}$	F: CCGGAATTCGCGGCCGCTTCTAGACGCTATGTAGCTCCA R: TGCACTGCAGCGGCCGCTACTAGTCACCAGGCCCGGAAG	**gDNA from *M. bovis* BCG Pasteur**
$\mathbf{P_{hspX}}$	F: CCGGAATTCGCGGCCGCTTCTAGACGCGTAAAGCACCCGATCCTT R: TGCACTGCAGGCGGCCGCTACTAGTTGGTGGCCATTTGATGCCTCCT	**gDNA from *M. bovis* BCG Pasteur**
$\mathbf{P_{hspXT}}$	F: CCGGAATTCGCGGCCGCTTCTAGAGCATGATCAACCTCCGCTGTTC R: TGCACTGCAGGCGGCCGCTACTAGTTGGTGGCCATTTGATGCCTCCT	**gDNA from *M. bovis*BCG Pasteur**
PL5X	F: GTTTCTTCGAATTCGCGGCCGCTTCTAGAGACAGCTATGACCATGATTACG R: GTTTCTTCCTGCAGCGGCCGCTACTAGTAATGCGATCTCCCTTTCC	**pL5 promoter** [18]
PL5Y[a]	Not applicable	**pL5 promoter** [18]
PL5Z[a]	Not applicable	**pL5 promoter** [18]
leuD	F: CCGGAATTCGCGGCCGCTTCTAGAATGGAAGCCTTTCAC R: TGCACTGCAGCGGCCGCTACTAGTTTATTATCAGGGGGCGGGTAGA	**pUP410** [19]
Ag85B[a]	Not applicable	**Commercial synthesis**
OriMt	F: CCGGAATTCGCGGCCGCTTCTAGACCCGACACCCGCTCCC R: TGCACTGCAGCGGCCGCTACTAGTCCAGCCCACCAGCTC	**Commercial synthesis**
kan$^{\mathbf{r}}$	F: CCGGAATTCGCGGCCGCTTCTAGAGATCAGTACTTTGTGT R: TGCACTGCAGCGGCCGCTACTAGTTTAGAAAAACTCATCG	**pUS977** [16]
egfp	F: CCCGAATTCGCGGCCGCTTCTAGAATGTGAGGAGATTCTCAATGACG R: GCACTGCAGCGGCCGCTACTAGTTCACTTGTAC	**pRSET-EmGFP (Invitrogen$^{\mathbf{TM}}$)**

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

2.8. Statistical analysis

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

3. Results

3.1. BioBrick-compatible mycobacterial parts

All sequences in the mycobacterial toolbox constructed in this study, including 10 different promoters, 2 mycobacterial coding

Table 2
Plasmids constructed in this study.

Plasmids	Description
pSB1C3/$\mathbf{P_{paN}}$	BioBrick submission vector & mycobacterial pAN promoter
pSB1C3/$\mathbf{P_{hsp60}}$	BioBrick submission vector & mycobacterial Hsp60 promoter
pSB1C3/$\mathbf{P_{18kDa}}$	BioBrick submission vector & mycobacterial 18 kDa promoter
pSB1C3/$\mathbf{P_{ag85B}}$	BioBrick submission vector & mycobacterial Ag85B promoter
pSB1C3/$\mathbf{P_{sag85B}}$	BioBrick submission vector & mycobacterial Ag85B promoter plus signal sequence
pSB1C3/$\mathbf{P_{hspX}}$	BioBrick submission vector & mycobacterial HspX promoter
pSB1C3/$\mathbf{P_{hspXT}}$	BioBrick submission vector & mycobacterial truncated HspX promoter
pSB1C3/PL5X	BioBrick submission vector & mycobacterial pL5X promoter
pSB1C3/PL5Y	BioBrick submission vector & mycobacterial pL5Y promoter
pSB1C3/PL5Z	BioBrick submission vector & mycobacterial pL5Z promoter
pSB1C3/*oriMt*	BioBrick submission vector & mycobacterial origin of replication
pSB1C3/*kan*	BioBrick submission vector & resistance gene to kanamycin
pSB1C3/*ag85B*	BioBrick submission vector & mycobacterial *ag85B* gene
pSB1C3/*leuD*	BioBrick submission vector & mycobacterial *leuD* gene
pUP500	BCG replicative and BioBrick-compatible vector, kan^{r}
pUP500/$\mathbf{P_{paN}}$	BCG replicative and BioBrick-compatible vector & mycobacterial pAN promoter
pUP500/$\mathbf{P_{hsp60}}$	BCG replicative and BioBrick-compatible vector & mycobacterial Hsp60 promoter
pUP500/$\mathbf{P_{18kDa}}$	BCG replicative and BioBrick-compatible vector & mycobacterial 18 kDa promoter
pUP500/$\mathbf{P_{ag85B}}$	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter
pUP500/$\mathbf{P_{sag85B}}$	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter plus signal sequence
pUP500/$\mathbf{P_{hspX}}$	BCG replicative and BioBrick-compatible vector & mycobacterial HspX promoter
pUP500/$\mathbf{P_{hspXT}}$	BCG replicative and BioBrick-compatible vector & mycobacterial truncated HspX promoter
pUP500/$\mathbf{P_{paN:\mathit{egfp}}}$	BCG replicative and BioBrick-compatible vector & mycobacterial pAN promoter upstream of *egfp* gene
pUP500/$\mathbf{P_{hsp60:\mathit{egfp}}}$	BCG replicative and BioBrick-compatible vector & mycobacterial Hsp60 promoter upstream of *egfp* gene
pUP500/$\mathbf{P_{18kDa:\mathit{egfp}}}$	BCG replicative and BioBrick-compatible vector & mycobacterial 18 kDa promoter upstream of *egfp* gene
pUP500/$\mathbf{P_{ag85B:\mathit{egfp}}}$	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter upstream of *egfp* gene
pUP500/$\mathbf{P_{sag85B:\mathit{egfp}}}$	BCG replicative and BioBrick-compatible vector & mycobacterial Ag85B promoter plus signal sequence upstream of *egfp* gene
pUP500/$\mathbf{P_{hspX:\mathit{egfp}}}$	BCG replicative and BioBrick-compatible vector & mycobacterial HspX promoter upstream of *egfp* gene
pUP500/$\mathbf{P_{hspXT:\mathit{egfp}}}$	BCG replicative and BioBrick-compatible vector & mycobacterial truncated HspX promoter upstream of *egfp* gene

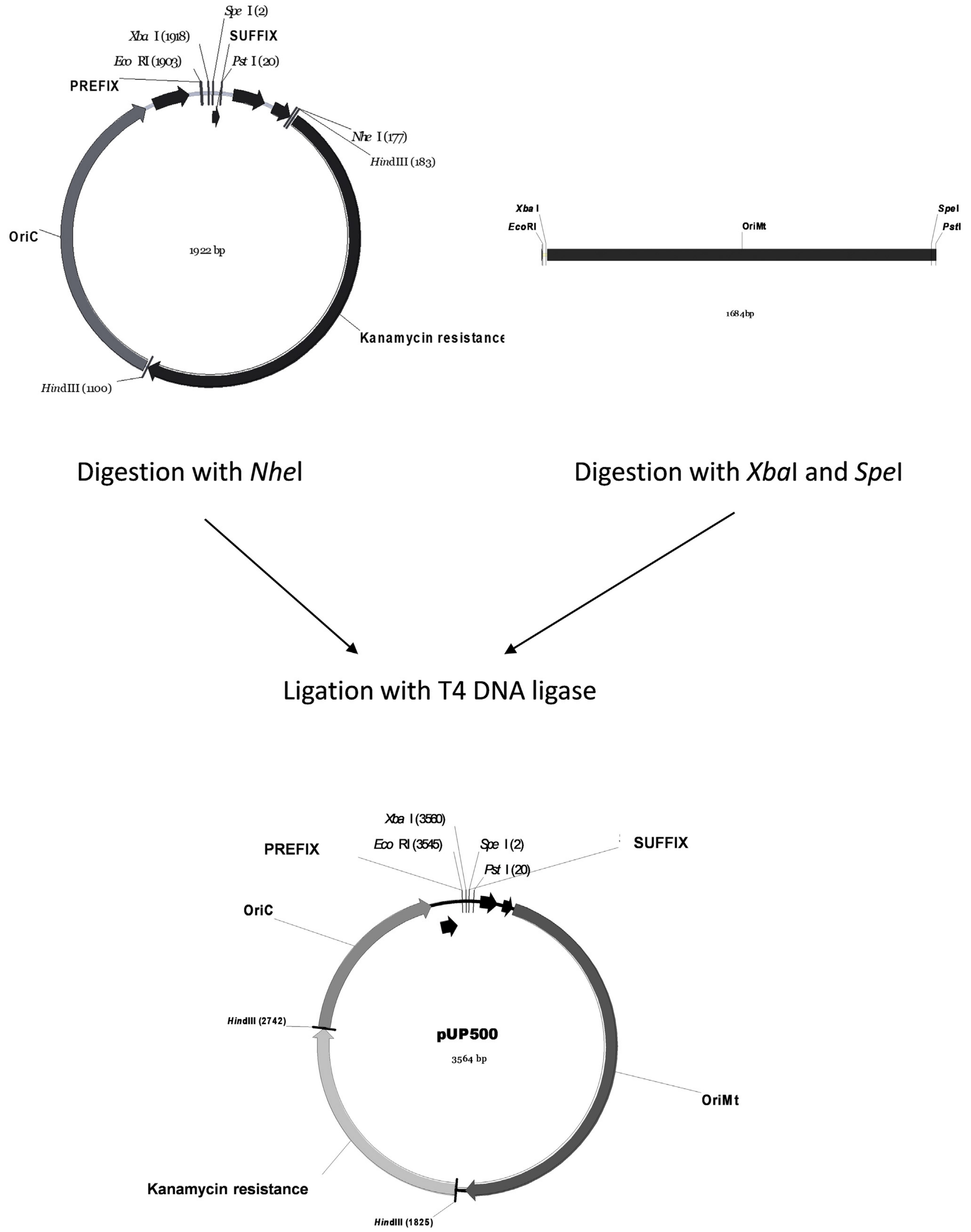

Fig. 1. Schematic outline of the construction of the base vector pUP500. The origin of replication for the vector pUP500 was commercially synthesized. The vector was digested with the enzyme *Nhe*I and the origin of replication was digested with *Xba*I and *Spe*I; the two molecules were ligated using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

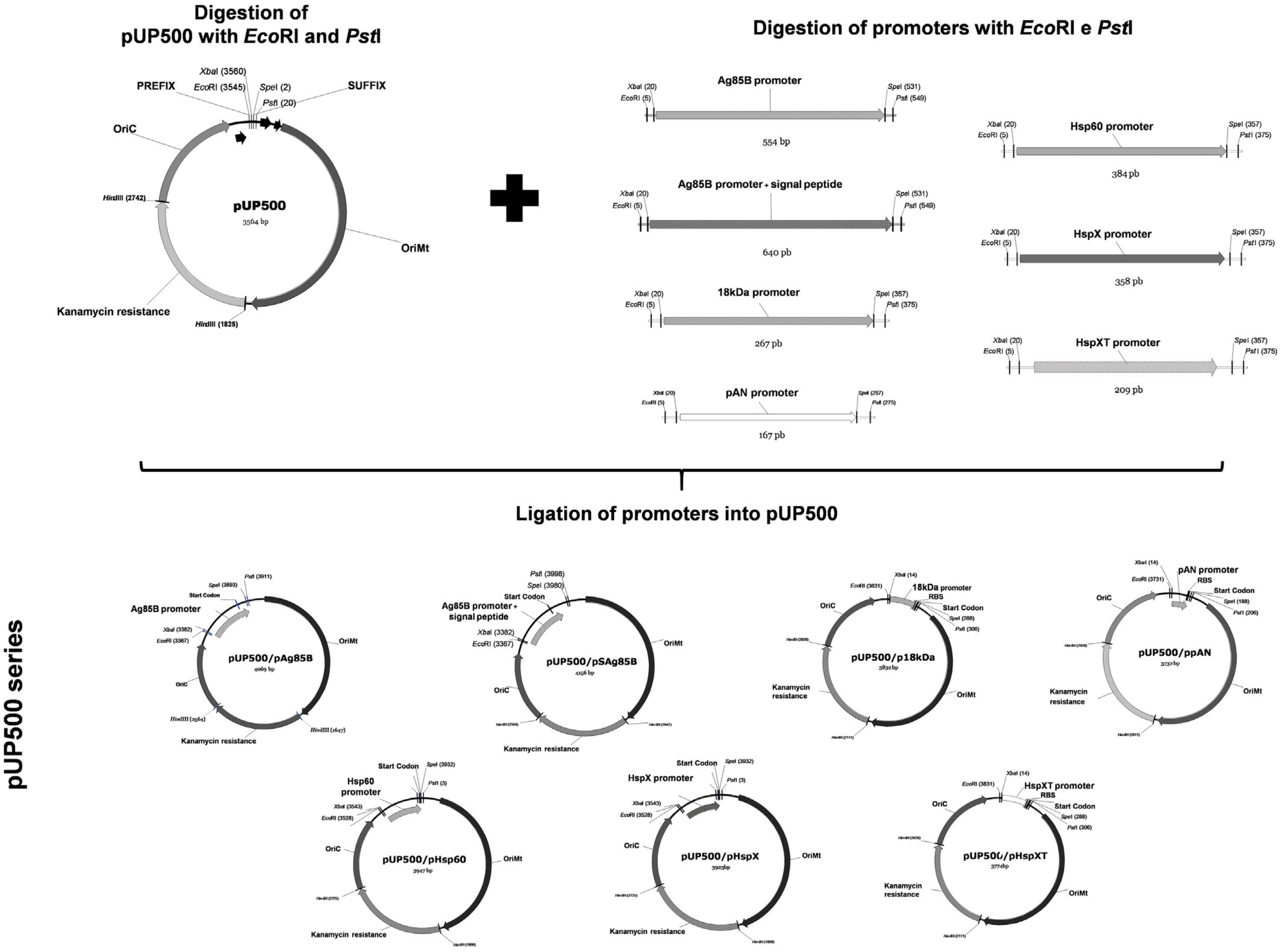

Fig. 2. Schematic outline of the pUP500 series of vectors constructed in this study. The pUP500 vector and the sequences of the mycobacterial promoters were digested with the *Eco*RI and *Pst*I enzymes; then, promoters were individually ligated into pUP500 using the enzyme T4 DNA ligase, generating the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

sequences, a reporter gene and an antibiotic resistance gene, were cloned as BioBrick parts into pSB1C3, the standard plasmid from the BioBricks Repository (Table 1). The fragments containing the restriction sites associated with the BioBrick RFC 10 standard had these sequences removed by commercial synthesis. The functionality of the origin of replication and resistance gene to kanamycin was demonstrated by visualization of colony growth in selective media with kanamycin (data not shown).

3.2. Construction of new expression vectors for mycobacteria

To obtain expression of heterologous antigens in rBCG according to BioBrick format, we constructed a base plasmid named pUP500, able to replicate in *E. coli* and *M. bovis* BCG, and containing a multiple cloning site with the prefix and suffix BioBrick sequences (Fig. 1). The pUP500 plasmid contains a kanamycin resistance gene flanked by restriction sites for *Hin*dIII, which allows deletion of the antibiotic selective marker. Then, seven different promoter sequences were individually cloned into pUP500, generating pUP500/P_{pAN}, pUP500/P_{18kDa}, pUP500/P_{hsp60}, pUP500/P_{ag85B}, pUP500/P_{sag85B}, pUP500/P_{hspX}, and pUP500/P_{hspXT} (Fig. 2). Transformation efficacy in BCG using these plasmids was low, ranging from 0,5–1 × 10^1 transformants/μg DNA. These vectors are available for expression of any antigen cloned according to the BioBrick format in BCG.

3.3. Characterization of mycobacterial promoters using egfp

Mutagenized promoters derived from pL5 (pL5X, pL5Y and pL5Z) had previously been constructed and characterized regarding their strength using eGFP as a reporter protein [18]. For this reason, we have only cloned these sequences as Biobrick parts in the pSB1C3 base vector (Table 2). All other promoter sequences of the pUP500 series of vectors were cloned into pUP500 and used to drive the expression of eGFP, further determined by fluorescence microscopy and flow cytometry (Fig. 3). In comparison to the wild-type BCG Pasteur, a higher percentage of fluorescent BCG cells was observed when using pAN or 18 kDa promoters, followed by HspX, the truncated sequence of HspX (HspXT), and Ag85B ($P < 0.05$). No difference was observed when Hsp60 or Ag85B plus signal sequence were used to drive expression of eGFP (Fig. 3C). When the intensity of fluorescence was determined for gated eGFP-positive BCG cells (Fig. 3D), these differences were maintained, except for the HspXT that did not differ from the control in this analysis ($P > 0.05$). When the HspX and HspXT promoter sequences were evaluated, we observed higher levels of eGFP expression driven by full sequence of the HspX promoter ($P < 0.05$), in comparison with the truncated version (HspXT). Also noteworthy was the fact that rBCG strains harboring pUP500 plasmids expressed eGFP inside macrophages at similar levels to those observed *in vitro* (Fig. 3B); whereas no fluorescence was detected in macrophages infected with *wt*BCG. Low fluorescence intensity was observed by fluorescence microscopy in

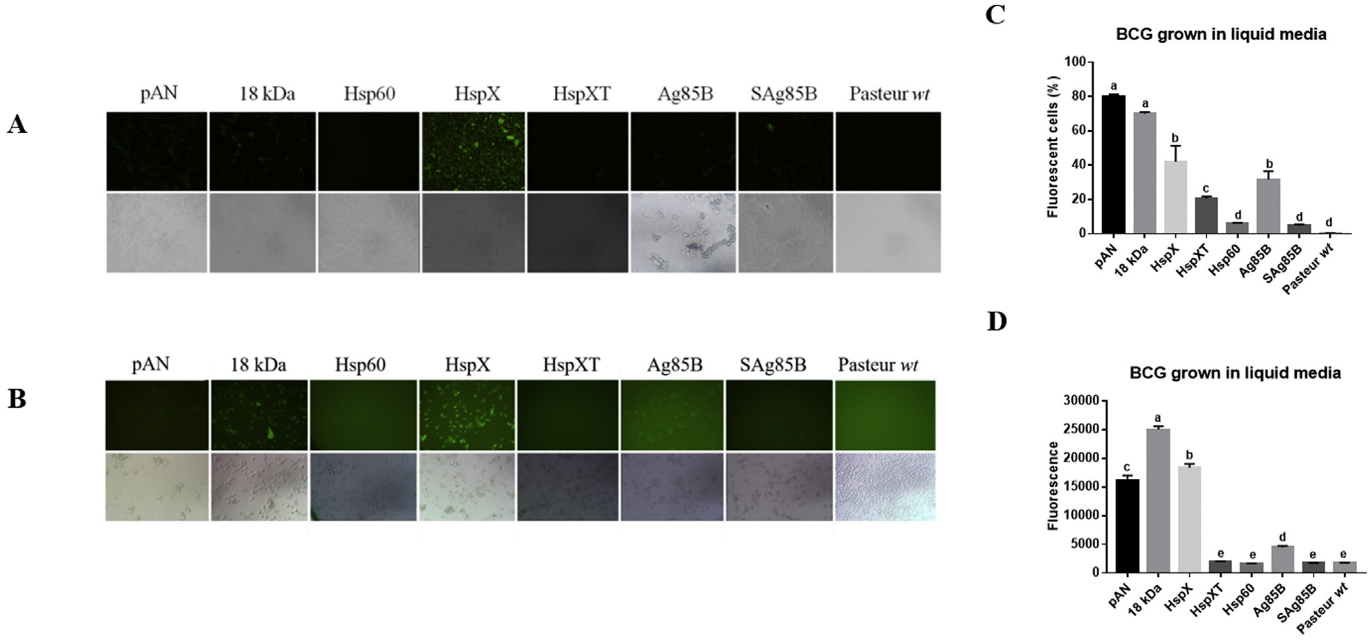

Fig. 3. Fluorescence detection induced by pUP500 plasmids in rBCG cultured *in vitro* and inside macrophages. The expression of *egfp* was driven by the upstream promoter sequences of the pUP500 series of vectors, as indicated, and confirmed by fluorescence microscopy (A and B) and flow cytometry (C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

macrophages infected with strains in which the promoters Hsp60, HspXT, and Sag85B were used to drive eGFP expression. In contrast, fluorescence levels were higher in macrophages than *in vitro* when the promoter 18 kDa was used to express the gene.

4. Discussion

Ins this study, we constructed a BioBrick mycobacterial toolbox containing ten different promoters, two mycobacterial coding sequences, a reporter gene, an antibiotic resistance gene and seven base episomal vectors. The expression of foreign antigens in a cell is driven by the use of different promoters, the activity of which determines antigen expression level and also affects the stability of the vaccine vector [13]. The ten different promoters that we constructed as BioBrick parts included three mutagenized promoters generated in a previous study [18] through error-prone PCR of the strong pL5 promoter (pL5X, pL5Y, and pL5Z), five classic (pAN, 18 kDa, Hsp60, Ag85B, and Sag85B), and two inducible promoters (HspX and HspXT). These classic promoters have been extensively used to drive expression of viral, bacterial, and parasitic antigens in BCG [13]. The truncated and whole sequences of the HspX promoter are differentially regulated in virulent or avirulent mycobacterial strains grown in liquid media, low-oxygen conditions, or inside macrophages [20,21]. Promoters pL5X, pL5Y, and pL5Z are mutated versions of the pL5 strong promoter, derived from mycobacteriophage L5, and were previously identified as having low, intermediate, and high strengths, respectively, when compared to the native promoter, using *egfp* as a reporter gene [18]. Here, the strength of classic and inducible promoters, as well as the functionality of these constructs, was determined through a systematic strategy using the same plasmid backbone and the same reporter gene under the control of each promoter.

The coding sequences to be expressed in our mycobacterial BioBrick toolbox include the mycobacterial sequences *ag85B* and *leuD* and the reporter gene *egfp*. The 85B antigen is part of a complex of proteins that play an important role in mycobacterial pathogenesis [22,23]. It has therefore been extensively evaluated as a vaccine candidate to improve BCG efficacy [24,25]. The *leuD* gene codes for an essential enzyme in leucine biosynthesis and has been used in auxotrophic complementation systems [19] to stabilize and make rBCG vaccine strains safer and without the need for antibiotic selection [26,27]. Moreover, we designed episomal vectors as they can deliver high levels of expression, although often at the cost of vector stability [15]. The pUP500 plasmid designed in our study allows the construction of auxotrophic BCG strains lacking the antibiotic resistance marker. We previously demonstrated that the leucine auxotroph system can be used to stably express foreign genes in mycobacteria without antibiotic selection *in vivo* [27] so we also developed BioBrick parts for this system in order to improve vaccine safety.

Structural and functional characteristics of the plasmid may affect copy number and vaccine stability due to the metabolic burden associated with maintaining it in the bacterial cell [15]. The use of a common plasmid backbone benefits the standardization and reproducibility of rBCG strains obtained and decreases both the bias of plasmid copy number in antigen expression variations and the unpredictability of vaccine efficacy. We used the eGFP as a reporter protein to assess the activity of different promoters in rBCG cultured *in vitro* and inside macrophages. This protein has mutations in two amino acids that result in higher levels of fluorescence than the wild-type GFP from jellyfish *Aequeora victoria* [28,29]. Kanno and colleagues previously demonstrated that measurement of fluorescence levels of eGFP by flow cytometry is a reliable tool to determine promoter activity, and it correlates with total amount of this protein in SDS-PAGE [18]. Besides, in a previous work developed by our group, other two proteins derived from *Leptospira interrogans* were expressed in rBCG using most of the vectors constructed in this study and characterized by *Western blot*, helping us to validate their functionality [30].

Several features of these experiments are noteworthy. Firstly, despite the fact that the Hsp60 gene promoter is considered to be a strong promoter that is widely used for expression of heterologous antigens in rBCG [13,15], we obtained no significant eGFP expression using this promoter. Kanno and colleagues also observed weak expression levels of GFP in *M. smegmatis* using the Hsp60 promoter, even when cells were exposed to heat shock treatment [18]. The result may be attributed to a high metabolic load imposed by eGFP synthesis controlled by this promoter. Other studies have reported structural instability of Hsp60, which can be associated with failure of a vectorized-BCG vaccine against leptospirosis [17,31]. The constructions pUP500/P_{hsp60} and pUP500/P_{sag85B} were already used to express antigens from *Leptospira interrogans* in BCG successfully, demonstrating their functionality [30].

Dokladda and colleagues also observed that HspX is induced in avirulent strains both *in vitro* and inside macrophages, while the HspXT promoter was activated only in virulent strains grown in macrophages or under conditions of low oxygen tension, corroborating with our cytometry data [20]. Considering these data, HspX could be considered a more suitable choice for the expression of vaccine antigens in avirulent/virulence-attenuated strains, such as BCG. Also, results of macrophage infection with rBCG using the 18 kDa promoter are in accordance with its higher activity during intracellular growth [32].

In conclusion, we have developed a series of Biobrick parts that can be used to obtain stable expression of foreign genes in BCG at different levels of expression. The functionality of these parts was demonstrated by flow cytometry and fluorescence microscopy, and the strength of promoters *in vitro* was evaluated in a systematic strategy. The technology we have applied to assemble sequences from mycobacteria will support other projects with recombinant BCG. Moreover, we provide a range of vectors for expression of foreign antigens in BCG. Although further work is required to provide more quantitative measures of expression levels, and to measure expression with a wider range of genes, the described Biobrick mycobacterial toolbox can be used for more efficient and controlled expression of foreign genes in BCG and other mycobacteria. We believe that our study will help the elucidation of the influence of molecular characteristics in the response induced by BCG-based vaccines in a more economical and practical manner than conventional cloning, as well as demonstrating the use of this kit applied to other pathogens.

Author contributions

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

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Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.07.002.

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