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A two-plasmid system for stable, selective-pressure-independent expression of multiple extracellular proteins in mycobacteria

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Recombinant mycobacteria expressing *Mycobacterium tuberculosis* extracellular proteins are leading candidates for new vaccines against tuberculosis and other mycobacterial diseases, and important tools both in antimycobacterial drug development and basic research in mycobacterial pathogenesis. Recombinant mycobacteria that stably overexpress and secrete major extracellular proteins of *M. tuberculosis* in native form on plasmids pSMT3 and pNBV1 were previously constructed by the authors. To enhance the versatility of this plasmid-based approach for mycobacterial protein expression, the *Escherichia coli*/mycobacteria shuttle plasmid pGB9 was modified to accommodate mycobacterial genes expressed from their endogenous promoters. Previous studies showed that the modified plasmid, designated pGB9.2, derived from the cryptic *Mycobacterium fortuitum* plasmid pMF1, was present at a low copy number in both *E. coli* and mycobacteria, and expression of recombinant *M. tuberculosis* proteins was found to be at levels paralleling its copy number, that is, approximating their endogenous levels. Plasmid pGB9.2 was compatible with the shuttle vectors pSMT3 and pNBV1 and in combination with them it simultaneously expressed the *M. tuberculosis* 30 kDa extracellular protein FbpB. Plasmid pGB9.2 was stably maintained in the absence of selective pressure in three mycobacterial species: *Mycobacterium bovis* BCG, *M. tuberculosis* and *M. smegmatis*. Plasmid pGB9.2 was found to be self-transmissible between both fast- and slow-growing mycobacteria, but not from mycobacteria to *E. coli* or between *E. coli* strains. The combination of two compatible plasmids in one BCG strain allows expression of recombinant mycobacterial proteins at different levels, a potentially important factor in optimizing vaccine potency.

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INTRODUCTION

Tuberculosis (TB), the first disease declared a global emergency by the World Health Organization (WHO), is a leading cause of death, especially in the developing world (Pablos-Mendez *et al.*, 1998). Exacerbating the problem is the worldwide emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis*, the primary causative agent of TB. This situation has created an urgent need for a vaccine against tuberculosis that is more efficacious than the current vaccine, and for new drugs to combat the disease (Cohn *et al.*, 1997).

Extracellular proteins of *M. tuberculosis* figure prominently in both new vaccine and drug development (Harth *et al.*, 1997). Extracellular proteins of *M. tuberculosis* are proteins that are secreted or otherwise released by the bacterium into

its extracellular milieu. In the human host, *M. tuberculosis* principally multiplies in mononuclear phagocytes, in which case extracellular proteins are released into a membrane-bound phagosome (Harth *et al.*, 1994, 1996; Lee & Horwitz, 1995).

In broth culture, *M. tuberculosis* releases large numbers of proteins into the medium; 12 of these proteins are present in especially large amounts (Horwitz *et al.*, 1995; Jungblut *et al.*, 1999). The major secretory protein of *M. tuberculosis*, a protein of 30 kDa molecular mass, is a member of a family of four related proteins, FbpA, B, C and D. Three of the four proteins, FbpA (antigen 85A, encoded by *fbpA*=Rv3804c), FbpB (antigen 85B, encoded by *fbpB*=Rv1886c) and FbpC (antigen 85C, encoded by *fbpC*=Rv0129c) form the 30–32 kDa complex of highly homologous mycolyl transferases (Belisle *et al.*, 1997; Cole *et al.*, 1998; Anderson *et al.*, 2001). The fourth protein of ~24 kDa, FbpD (antigen Mpt51, encoded by *fbpD*=Rv3803c), is related to FbpA, B and C, but it remains unclear whether the protein is an enzymically active mycolyl

The GenBank accession number for the sequence of plasmid pGB9.2 is AY266291.

transferase. The relative activities of the three proteins FbpA, B and C *in vivo* are also not known.

The 30 kDa protein is of particular interest in new vaccine and drug development. With respect to vaccine development, immunization of guinea pigs with the 30 kDa protein induces substantial protective immunity against aerosol challenge with the highly virulent Erdman strain of *M. tuberculosis* (Horwitz *et al.*, 1995). Moreover, immunization of guinea pigs with a recombinant BCG overexpressing this protein (rBCG30) induces protective immunity superior to conventional BCG vaccine (Horwitz *et al.*, 2000; Horwitz & Harth, 2003). The rBCG30 vaccine is currently in Phase I human trials. With respect to drug development, the *M. tuberculosis* 30 kDa protein and the other two highly homologous mycolyl transferases of 32 kDa molecular mass are leading drug targets. Targeting the proteins' gene transcripts (mRNAs of *fbpA*, *fbpB* and *fbpC*), we have previously shown that antisense phosphorothioate oligonucleotides strongly inhibit *M. tuberculosis* growth in broth culture (Harth *et al.*, 2002). An analysis of the three-dimensional structure of the 30 kDa protein suggested a class of chemical inhibitors (Anderson *et al.*, 2001), and certain trehalose analogues aimed at the mycolyl transferase complex have been shown to inhibit *M. tuberculosis* growth (Rose *et al.*, 2002; G. Harth, B. Smith, M. Jung & M. A. Horwitz, unpublished results).

A key feature of the rBCG30 vaccine is the use of a plasmid (pMTB30) to overexpress the *M. tuberculosis* 30 kDa protein. Indeed, it is the overexpression of this protein which is essential to the enhanced efficacy of this vaccine (Horwitz *et al.*, 2000). The overexpression of multiple *M. tuberculosis* extracellular proteins in BCG or other mycobacterial hosts may result in even more potent vaccines. This may be accomplished by using a single plasmid containing multiple genes cloned in various orientations or by using two or more compatible plasmids. An advantage of the latter approach is that the relative expression of the proteins from different plasmids can be modulated according to plasmid copy number.

In this report, using the 30 kDa protein as an example, we describe the use of plasmid pGB9.2 to overexpress *M. tuberculosis* extracellular proteins in mycobacteria. Plasmid pGB9.2 is within the same compatibility group as pJAZ plasmids (Bachrach *et al.*, 2000). We here present the entire DNA sequence of the plasmid and demonstrate that: (a) pGB9.2 stably expresses the *M. tuberculosis* 30 kDa protein in mycobacteria in the absence of selective pressure; (b) pGB9.2 and a second compatible plasmid simultaneously express the 30 kDa protein in mycobacteria; (c) pGB9.2 is present and stably maintained at a low copy number allowing the intentional expression of selected proteins at a lower level than proteins expressed on a high-copy-number plasmid; (d) a 1.3 kb region of the plasmid is necessary for plasmid stability in mycobacteria; and (e) the plasmid is self-transmissible between fast- and slow-growing mycobacteria, but not from mycobacteria to *Escherichia coli*.

METHODS

Bacterial strains, antibiotics and plasmids. The strains, antibiotics and plasmids used in this study are listed in Table 1. *E. coli* strains were incubated for 24 h, *Mycobacterium smegmatis* strains for 3–5 days, and *Mycobacterium bovis* and *M. tuberculosis* strains for 2–3 weeks, all at 37 °C, and in the case of the mycobacteria in a 5% CO₂/95% air atmosphere.

Introduction of modifications in plasmid pGB9. The shuttle plasmid pGB9 was modified in its *E. coli* portion by eliminating 2.8 kb of non-essential regions, including the *camF* gene, a partial *tet^r* gene and part of the intergenic region of pACYC184 between the *tet^r* gene and the replication origin, *ori* p15A. The altered version of pGB9 was designated pGB9.2 (Fig. 1). Another deletion eliminated the two P_{lacZ} and *lacZ*α-carrying *AseI*–*AseI* fragments, which were replaced by a multi-cloning site.

Construction of plasmid pGB9.2 expressing the *M. tuberculosis* 30 kDa protein. The modified pGB9 plasmid pGB9.2 was assayed for its capacity to express recombinant proteins by inserting the *M. tuberculosis* *fbpB* gene into the plasmid's multi-cloning site and driving expression of the protein by its endogenous promoter. The 30 kDa protein gene cassette, consisting of the *fbpB* coding region preceded by ~500 bp of upstream DNA sequence, was inserted into the *PacI* site of pGB9.2 (Fig. 1).

Modifications of plasmid pNBV1 for conjugation assays. The mycobacterial shuttle vector pNBV1(Hyg^r) was modified by inserting the gene encoding apramycin resistance between the plasmid's two *DraI* sites to yield plasmid pNBV1(Apr^rHyg^r).

Expression of recombinant *M. tuberculosis* 30 kDa protein. Supernates from late-exponential-phase cultures (4 days for *M. smegmatis* 1-2c; 14 days for *M. bovis* BCG Tice and *M. tuberculosis* Erdman) were filtered through 0.45 and 0.22 μm acetate filters, then concentrated in Amicon Diaflo filtration units to a final concentration of 1 × 10⁸ cell equivalents μl⁻¹ and analysed for secreted 30 kDa protein on 12.5% denaturing polyacrylamide gels. Protein patterns were assessed densitometrically either directly or after immunoblotting using polyvalent rabbit anti-30 kDa protein antibodies and an enhanced chemiluminescent detection kit. The scanned gels and immunoblots were digitized with Adobe Photoshop software and the amounts of 30 kDa protein were expressed in arbitrary units using the NIH Image 1.62 software program. Baseline expression was the amount of endogenous 30 kDa protein expressed by the wild-type mycobacterial strain.

Assessment of plasmid stability. Stability of the plasmids pGB9.2 and pGB9.2-30 was assayed in *M. bovis* BCG Tice and *M. smegmatis* 1-2c by culturing a total of three independent plasmid-harboring clones in 7H9 broth without kanamycin for 30 generations (30 days for BCG and 5 days for *M. smegmatis*) and then enumerating c.f.u. of both strains after plating on 7H11 agar medium containing or lacking kanamycin (20 μg ml⁻¹). Genomic and plasmid DNA from strains harbouring plasmid pGB9.2 were quantified densitometrically (Jacobs *et al.*, 1991; Pushnova *et al.*, 2000). In addition, total DNA (various amounts) was transformed into DH5α, and kanamycin-resistant *E. coli* bacteria were enumerated to compare the transformation efficiency of pGB9.2 with that of pMTB30 in *E. coli*.

Assessment of self-transmissibility of pGB9. For bacterial mating experiments involving various *E. coli* and/or mycobacterial strains, donor and recipient bacteria were mixed on an Amicon YM3-43 filter at ratios of 1:1 or 1:10 and incubated for either 4 h or 16 h at 37 °C. Bacteria were washed with 1–2 ml of medium (Luria Bertani or 7H9) and exconjugants were evaluated after plating and incubation on selective medium.

Table 1. Bacterial strains, antibiotics and plasmids

Bacterial strains	Description	Reference
<i>E. coli</i> DH5 α	<i>recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻ m_K⁺) supE44 relA1 lacZx⁺</i>	Invitrogen
<i>E. coli</i> S17	λ pir ⁺ <i>recA thi pro hsdR⁻M⁺</i> [pLC28(Cam ^r Str ^s)]	Cheng <i>et al.</i> (1997)
<i>E. coli</i> SM10	λ pir ⁺ Cam ^s Str ^r	Cheng <i>et al.</i> (1997)
<i>E. coli</i> XL-1 Blue	<i>recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lac[F' proAB lacF^rZΔM15 Tn10(Tet^r)]</i>	Stratagene
<i>E. coli</i> Y1090	Δ (<i>lac</i>)U169 Δ lon <i>araD139 strA supF mcrA trpC22::Tn10(Tet^r)</i> [pMC9(Amp ^r Tet ^r)]	Stratagene
<i>M. bovis</i> BCG Tice		Organon
<i>M. smegmatis</i> 1-2c		Herrmann <i>et al.</i> (1996)
<i>M. tuberculosis</i> Erdman		ATCC 35801
<i>M. tuberculosis</i> Erdman	<i>glnA1::Tn5 aph(Km^r)</i>	Tullius <i>et al.</i> (2003)
<i>M. tuberculosis</i> Erdman	(Hyg ^r ; unmapped insertion)	Unpublished results
Antibiotics		
Ampicillin (Amp)	For <i>E. coli</i> , 100 μ g ml ⁻¹ ; for mycobacteria, 20 μ g ml ⁻¹	
Apramycin (Apr)	For <i>E. coli</i> , 50 μ g ml ⁻¹ ; for mycobacteria, 30 μ g ml ⁻¹	
Chloramphenicol (Cam)	For <i>E. coli</i> , 30 μ g ml ⁻¹	
Hygromycin (Hyg)	For <i>E. coli</i> , 250 μ g ml ⁻¹ ; for mycobacteria, 50 μ g ml ⁻¹	
Kanamycin (Kan)	For <i>E. coli</i> , 50 μ g ml ⁻¹ ; for mycobacteria, 20 μ g ml ⁻¹	
Pyrazinamide (Pza)	For mycobacteria, 50 μ g ml ⁻¹	
Streptomycin (Str)	For <i>E. coli</i> , 30 μ g ml ⁻¹	
2-Thiophene carboxylic acid hydrazide (TCH)	For mycobacteria, 5 μ g ml ⁻¹	
Tetracycline (Tet)	For <i>E. coli</i> , 12.5 μ g ml ⁻¹	
Plasmids		
pGB9	<i>E. coli</i> -mycobacteria shuttle vector; Kan ^r	Bachrach <i>et al.</i> (2000)
pLC28	Conjugative R6K derivative; Cam ^r Str ^s	Cheng <i>et al.</i> (1997)
R6K	Self-transmissible <i>E. coli</i> plasmid; Amp ^r Str ^r	ATCC
pMTB30	pSMT3:: <i>M. tuberculosis</i> Erdman <i>fbpB</i>	Harth <i>et al.</i> (1997)
pNBV1	<i>E. coli</i> -mycobacteria shuttle vector; Hyg ^r	Howard <i>et al.</i> (1995)
pSMT3	<i>E. coli</i> -mycobacteria shuttle vector; Hyg ^r	Herrmann <i>et al.</i> (1996)
pVK173T	<i>E. coli</i> -mycobacteria shuttle vector; Amp ^r Apr ^r Hyg ^r	Paget & Davies (1996)

RESULTS

DNA sequence features and coding capacity of pGB9 and generation of pGB9.2

The original plasmid pGB9 (Bachrach *et al.*, 2000) was composed of a 9.2 kb mycobacterial portion derived from the *M. fortuitum* plasmid pMF1 and a 5.6 kb portion derived from various *E. coli* plasmids for a total size of 14 841 bp. The mycobacterial portion remains mainly unmapped and very little is known about its coding capacity. One of the *Hind*III restriction fragments within the 9.2 kb mycobacterial DNA sequence contains the origin of replication and encodes a protein of the Rep family of replication proteins required for plasmid replication (Bachrach *et al.*, 2000).

To make pGB9 more amenable to genetic manipulations, we trimmed the *E. coli* portion of the plasmid to just the gene conferring kanamycin resistance and the replication origin *ori* p15A, and introduced into the now unique *Ase*I site a multi-cloning site containing the following unique

restriction enzyme sites: *Pac*I, *Spe*I, *Swa*I, *Eco*RV and *Nde*I. The final version of the modified plasmid was ~11.441 kb in size and was designated pGB9.2 (Fig. 1). Based on the known DNA sequences of the IS50L element and the newly introduced multi-cloning site, we established partial DNA sequences for both the *E. coli* and the mycobacterial portion of pGB9.2; the remaining portions were determined commercially (Sequetech). Of note was the difference in G+C content between the *E. coli* (~50 mol%) and the mycobacterial portion (~66 mol%). The p15A *ori* region places the plasmid in the low-copy-number category (~10–15 copies in *E. coli*); based on total DNA preparations and *E. coli* transformations, this is also true for the pGB9.2 copy number in mycobacteria (approximately one to two copies). Although transformation of mycobacteria with plasmids carrying the kanamycin resistance gene is sometimes unsuccessful (Garbe *et al.*, 1994), we did not have difficulty transforming *M. bovis* BCG, *M. smegmatis* or *M. tuberculosis* with pGB9.2. The mycobacterial portion (pMF1) is cryptic in all three mycobacterial species as well as in *Mycobacterium fortuitum*, from which it originated.

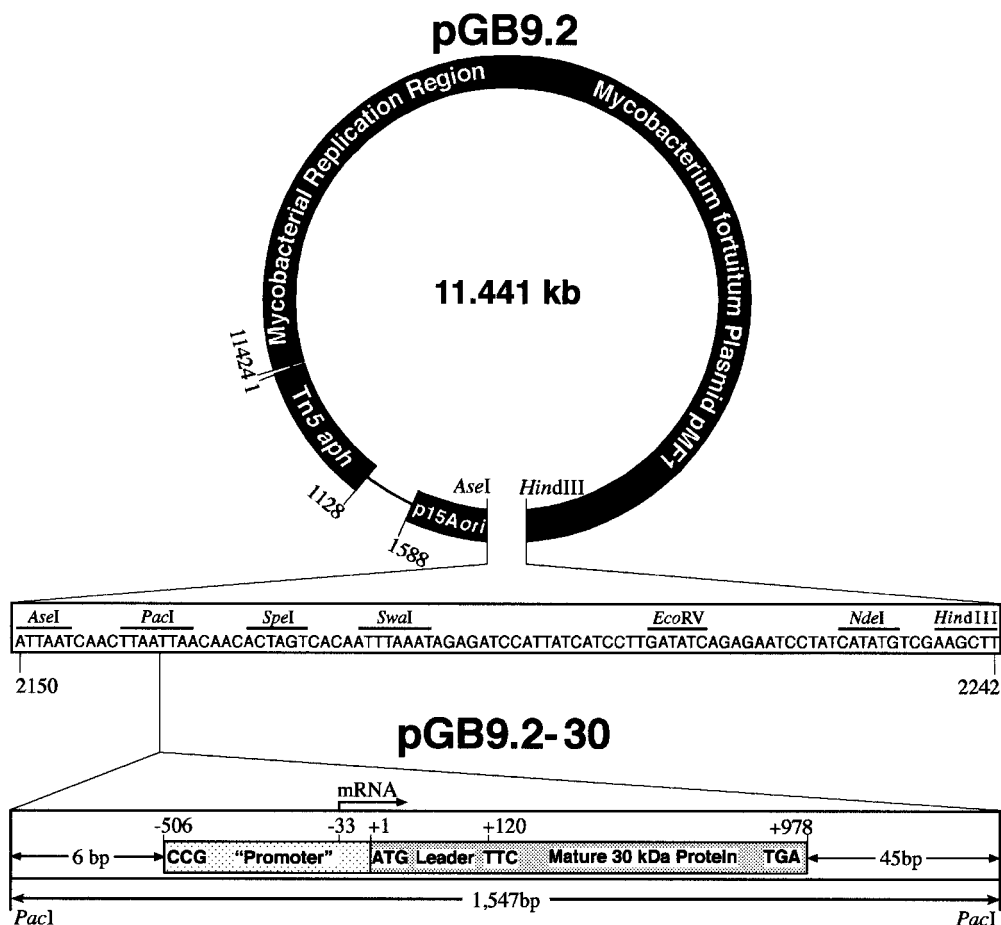


Fig. 1. Maps of plasmid pGB9.2 and its derivative pGB9.2-30. The shuttle vector pGB9.2 is composed of an *E. coli* portion, containing the kanamycin resistance gene from Tn5 (Tn5aph) and an origin of replication region from plasmid pACYC184 (p15A ori), and a mycobacterial portion, containing the entire *M. fortuitum* plasmid pMF1 with its mycobacterial replication region. The map also shows the DNA sequence of the multi-cloning site flanked by AseI and HindIII sites. The recombinant shuttle plasmid pGB9.2-30 contains the *M. tuberculosis* Erdman *fbpB* gene inserted into the multi-cloning cassette's PacI site. For convenience, the *fbpB* gene including its relevant functional features is shown in a left-to-right orientation, although the analysed clones were oriented in the opposite direction. The leader peptide spans the first 40 amino acids. The 6 bp stretch at the 5' end of the *fbpB* cassette is a HindIII site and the 45 bp stretch at the 3' end is from the *fbpB* genomic locus immediately downstream of the gene's stop codon.

Expression of a major *M. tuberculosis* extracellular protein by pGB9.2

We first assessed expression of recombinant 30 kDa protein in the fast-growing mycobacterium *M. smegmatis*. The recombinant mycobacterial strain harbouring one to two copies of the plasmid expressed only about two- to threefold the amount of 30 kDa protein that the wild-type strain expressed (30 kDa protein homologue in *M. smegmatis* is ~27 kDa). In contrast, recombinant *M. smegmatis* carrying the plasmid pMTB30, which is present at a higher copy number, expressed five- to sixfold more 30 kDa protein than the wild-type strain (Horwitz *et al.*, 2000). Thus, the magnitude of expression is highly correlated with copy number. Next, we generated recombinant slow-growing mycobacteria, BCG Tice and *M. tuberculosis* Erdman, to

determine if expression of recombinant 30 kDa protein is different from that observed in *M. smegmatis*. Again, the plasmid copy number was relatively low, one to two copies, and the recombinant strains expressed only about two- to threefold the amount of 30 kDa protein that the wild-type strains expressed, while pMTB30 carrying BCG Tice and *M. tuberculosis* expressed five- to sixfold more 30 kDa protein than the wild-type strains (Horwitz *et al.*, 2000; this study). Most of the expressed 30 kDa protein was secreted in all mycobacterial strains, and protein expression was quantified by scanning gels and immunoblots (Fig. 2, Table 2). As a control, we assessed expression of recombinant 30 kDa protein in *E. coli*, where we could not detect any expression of this protein, presumably due to the incompatibility of mycobacterial promoters and the *E. coli* transcription machinery.

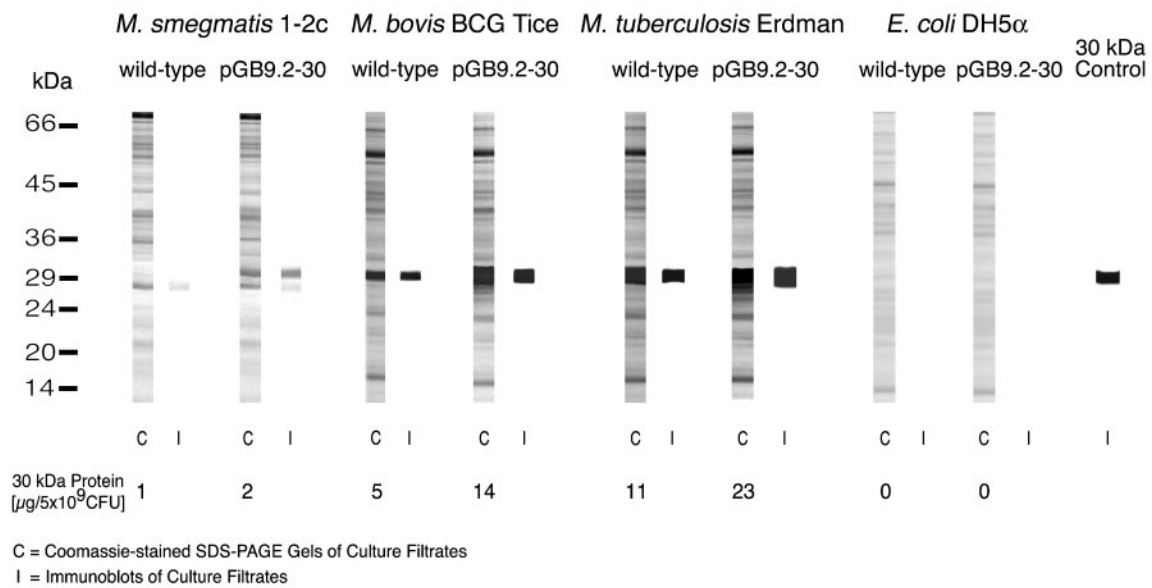
Secretion of pGB9.2-30 Expressed *M. tuberculosis* 30 kDa Protein in Various Mycobacteria

Fig. 2. Secretion of *M. tuberculosis* 30 kDa protein expressed from pGB9.2-30 in various mycobacteria. Concentrated culture filtrates were subjected to gel electrophoresis (using 5×10^9 c.f.u.) or to gel electrophoresis and immunoblotting (using 5×10^8 c.f.u.). Coomassie-stained gels (C) and immunoblots (I) were digitized and the images were analysed to yield the indicated amounts of secreted 30 kDa protein. The 'control' lane contains 10 μ g of recombinant *M. tuberculosis* 30 kDa protein purified from *M. smegmatis* 1-2c.

Expression of a major *M. tuberculosis* extracellular protein by pGB9.2 and a compatible second plasmid

One approach to generating recombinant BCG strains expressing various *M. tuberculosis* proteins involves placing two or more compatible plasmids in BCG. We investigated the two-plasmid approach for the pMTB30-carrying strains *M. bovis* BCG Tice and *M. tuberculosis* Erdman, both of which expressed five- to sixfold more 30 kDa protein than the wild-type strains (Horwitz *et al.*, 2000; this study). After introduction of the recombinant plasmid pGB9.2-30 into BCG Tice and *M. tuberculosis* Erdman, expression of 30 kDa protein was additive in

both strains (Fig. 3, Table 2), underscoring the usefulness of this two-plasmid expression approach. For *M. bovis* BCG, 1 l of bacterial culture ($\sim 5 \times 10^{11}$ c.f.u.) yielded approximately 0.5 mg protein, increasing about five- to sixfold in the presence of pMTB30 and eightfold in the presence of the two plasmids pMTB30 and pGB9.2-30. The same result was observed in *M. tuberculosis*, which typically showed the highest level of 30 kDa protein expression. While the relative increase in expression of recombinant 30 kDa protein in *M. tuberculosis* was similar to the increase observed in BCG, the absolute amount of expressed and secreted 30 kDa protein was always higher in *M. tuberculosis* than in BCG, because wild-type *M.*

Table 2. Secretion of endogenous and recombinant *M. tuberculosis* 30 kDa protein in various mycobacteria

Protein levels, shown in mg l^{-1} , were measured from scanned gels and immunoblots digitized by Adobe Photoshop 5.5 and analysed using NIH Image 1.62 software programs.

Species	Endogenous 30 kDa protein*	Endogenous + recombinant 30 kDa protein				
		pMTB30	pNBV1-30	pGB9.2-30	pMTB30 + pGB9.2-30	pNBV1-30 + pGB9.2-30
<i>M. smegmatis</i>	0.1	0.5	0.6	0.2	0.7	0.8
<i>M. bovis</i> BCG	0.5	2.8	3.4	1.4	4.2	4.8
<i>M. tuberculosis</i>	1.1	6.5	8.3	2.3	8.8	10.6
<i>E. coli</i>	0	0	0	0	0	0

*30 kDa protein (FbpB; mycolyl transferase) in *M. smegmatis* ~ 27 kDa; in BCG and *M. tuberculosis* ~ 30 kDa.

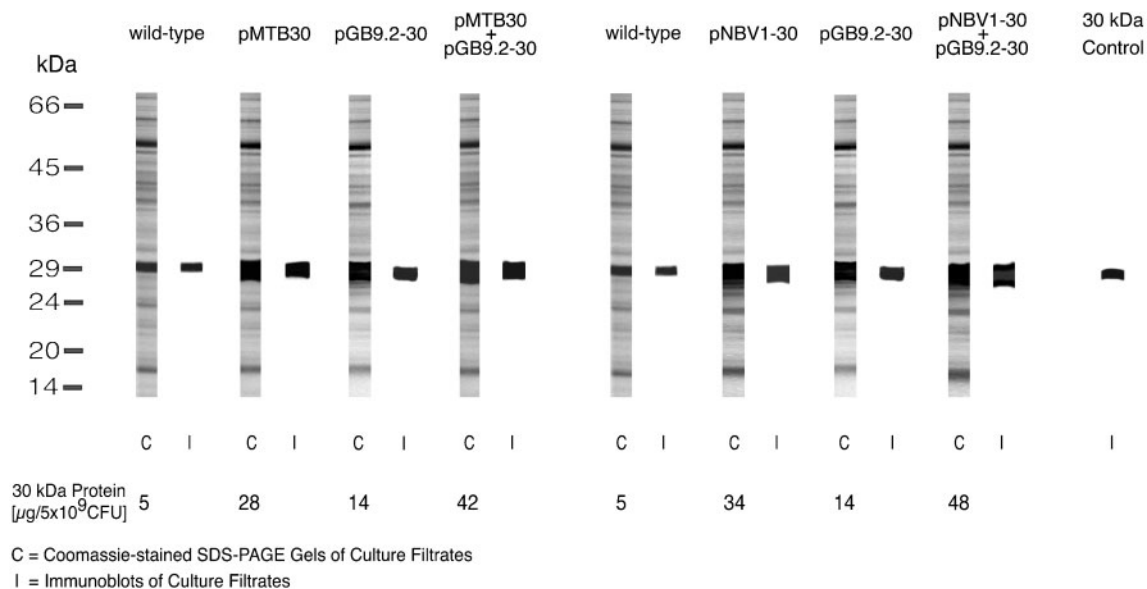
Two-Plasmid System for the Expression of *M. tuberculosis* 30 kDa Protein in *M. bovis* BCG Tice

Fig. 3. Expression and secretion of *M. tuberculosis* 30 kDa protein in recombinant BCG carrying two plasmids. Concentrated culture filtrates were subjected to gel electrophoresis (using 5×10^9 c.f.u.) or to gel electrophoresis and immunoblotting (using 5×10^8 c.f.u.). Stained gels (C) and immunoblots (I) were digitized and the images analysed to yield the indicated amounts of secreted 30 kDa protein. The 'control' lane contains 10 μg of recombinant *M. tuberculosis* 30 kDa protein purified from *M. smegmatis* 1-2c.

tuberculosis cells express about twofold the amount of endogenous 30 kDa protein that BCG cells express.

Stability of pGB9.2 in mycobacteria

For pGB9.2 to be useful in our vaccine protocols, it was mandatory that it be retained in recombinant BCG bacteria so as to provide continuously expressed recombinant *M. tuberculosis* 30 kDa protein. Stability of plasmid pGB9.2-30 was assayed in *M. bovis* BCG Tice and *M. smegmatis* 1-2c by culturing the bacteria in medium without kanamycin for 30 generations and then evaluating c.f.u. on medium containing or lacking kanamycin. The enumeration of the plated serial dilutions of three independent assays revealed that the c.f.u. counts were very similar: the mean count of three assays for BCG on kanamycin-containing medium was $(7.8 \pm 0.03) \times 10^7$ c.f.u. ml^{-1} and on kanamycin-lacking medium $(7.8 \pm 0.2) \times 10^7$ c.f.u. ml^{-1} ; the corresponding numbers for *M. smegmatis* were $(5.7 \pm 0.1) \times 10^8$ c.f.u. ml^{-1} and $(5.8 \pm 0.2) \times 10^8$ c.f.u. ml^{-1} . These results clearly demonstrate that pGB9.2-30 is stably maintained by >95% of all bacteria in a culture over many generations.

To prove that the plasmid was recoverable, we quantified the two plasmids, pGB9.2 and pGB9.2-30, by agarose gel assays and bacterial transformations. From a total of $\sim 5 \times 10^9$ c.f.u., we isolated $\sim 20 \mu\text{g}$ of DNA which contained $\sim 70 \text{ ng}$ of pGB9.2, as determined by gel scans. Since

one pGB9.2 plasmid equals $\sim 1.2 \times 10^{-8}$ ng DNA, we expected $\sim 60 \text{ ng}$ pGB9.2 DNA in 5×10^9 bacteria if each bacterium carried one copy of pGB9.2. Transformations of purified plasmid DNA and total bacterial DNA from rBCG Tice-pGB9.2 into *E. coli* DH5 α yielded kanamycin-resistant *E. coli* bacteria at mean frequencies of $\sim 10^6$ transformants per μg pGB9.2 plasmid DNA and 5000 transformants per μg total bacterial DNA. Using the values mentioned above, we expected 1 μg total DNA to contain $\sim 3 \text{ ng}$ pGB9.2 DNA, which would result in ~ 3000 transformants. This result shows that the recombinant BCG strain most likely harbours one to two copies of pGB9.2. Values for the plasmid pGB9.2-30 were also consistent with this copy number. The same analyses were performed for pMTB30 ($\sim 10 \text{ kb}$), the results of which demonstrated that recombinant BCG bacteria harbouring pMTB30 contain ~ 13 – 14 copies of pMTB30. The integrity of the plasmids pGB9.2, pGB9.2-30 and pMTB30 was demonstrated by recovering the plasmids from transformed *E. coli* clones and digesting them with several restriction endonucleases.

Based on previously published data, we knew that a 4.2 kb *Hind*III restriction fragment of pMF1 contains the mycobacterial replication region (Bachrach *et al.*, 2000). Hence, we considered introducing changes in a location of pMF1 as far removed from the *ori* region as possible by deleting a 1.3 kb fragment flanked by *Hind*III and *Xba*I restriction sites. This deletion mutant grew well in *E. coli*, but poorly

in *M. smegmatis* where the plasmid was lost by apparently all cells over a 30-generation growth period, since no kanamycin-resistant clones were obtained upon shifting bacteria from medium lacking kanamycin to medium containing the antibiotic. Hence, the 1.3 kb region was necessary for plasmid stability in mycobacteria in the absence of selective pressure.

Bacterial conjugations to assess self-transmissibility of pGB9.2

In a previous report, we showed that the plasmid pSMT3 is neither self-transmissible nor mobilizable, regardless of the bacterial species in which it resides (Horwitz & Harth, 2003). However, we expected this not to be the case for pGB9.2 because it has a much greater coding capacity and belongs to a different compatibility group. To assess the mobility of plasmid pGB9.2(Kan^r), we performed a series of bacterial conjugations and compared its mobility with that of pNBV1(Apr^rHyg^r) and pSMT3(Hyg^r) (Table 3). Transmissibility was analysed by investigating the transfer of pGB9.2 from *M. smegmatis* 1-2c to the wild-type mycobacterium *M. tuberculosis* Erdman. Exconjugants, plated on medium containing 2-thiopene carboxylic acid hydrazide

and kanamycin, arose at a frequency of 5.1×10^{-7} , demonstrating that pGB9.2 is self-transmissible between mycobacterial strains. The genetic background of the recipient mycobacterium does not influence the mobility of pGB9.2 since mixing of *M. smegmatis* 1-2c carrying pGB9.2 with either *M. bovis* BCG Tice[pSMT3(Hyg^r)] or *M. tuberculosis* Erdman[pNBV1(Apr^rHyg^r)] yielded two-plasmid-carrying BCG and *M. tuberculosis* clones at frequencies of 2.7 and 1.8×10^{-7} , respectively. In contrast, the small shuttle vectors pSMT3 and pNBV1 were not self-transmissible and could not be mobilized by plasmid pGB9.2.

As a control, we assessed the mobility of the three plasmids, pGB9.2, pNBV1 and pSMT3, between *E. coli* strains as well as between *E. coli* and mycobacterial strains. These assays demonstrated that the three plasmids cannot be introduced into *E. coli* strains by conjugational transfer. Even the presence in an *E. coli* donor strain of the self-transmissible plasmid R6K did not result in the transfer of any of the three mycobacterial shuttle vectors. However, the *E. coli*/mycobacteria shuttle vector pVK173T, which carries a functional origin of transfer, can be mobilized from *E. coli* to mycobacteria with the help of plasmid R6K.

Table 3. Frequencies of conjugational plasmid transfers in various bacterial species

Donors	Recipients	Selection (markers/plasmid)	10 ⁷ × Frequency
<i>M. smegmatis</i> 1-2c			
pGB9.2(Kan ^r)	<i>M. tuberculosis</i> wild-type	TCH ^r , Kan ^r /pGB9.2	5.1
	<i>M. bovis</i> BCG [pSMT3(Hyg ^r)]	Hyg ^r , Pza ^r , Kan ^r /pGB9.2	2.7
	<i>M. tuberculosis</i> [pNBV1(Apr ^r Hyg ^r)]	Apr ^r , Hyg ^r , TCH ^r , Kan ^r /pGB9.2	1.8
pNBV1(Apr ^r Hyg ^r)	<i>M. tuberculosis gln</i> A1 :: Km ^r	Kan ^r , TCH ^r , Apr ^r , Hyg ^r /pNBV1	0
pSMT3(Hyg ^r)	<i>M. tuberculosis gln</i> A1 :: Km ^r	Kan ^r , TCH ^r , Hyg ^r /pSMT3	0
pGB9.2(Kan ^r) + pSMT3(Hyg ^r)	<i>M. tuberculosis</i> wild-type	TCH ^r , Kan ^r /pGB9.2	2.3
	<i>M. tuberculosis</i> wild-type	TCH ^r , Hyg ^r /pSMT3	0
pVK173T(Amp ^r Apr ^r Hyg ^r)	<i>M. bovis</i> BCG wild-type	Apr ^r , Hyg ^r /pVK173T	0
	<i>M. tuberculosis</i> wild-type	Apr ^r , Hyg ^r /pVK173T	0
<i>E. coli</i> DH5α			
pGB9.2(Kan ^r)	<i>E. coli</i> XL-1 Blue(Tet ^r)	Tet ^r , Kan ^r /pGB9.2	0
	<i>E. coli</i> Y1090 [pMC9(Amp ^r Tet ^r)]	Tet ^r , Kan ^r /pGB9.2	0
pNBV1(Apr ^r Hyg ^r)	<i>E. coli</i> XL-1 Blue(Tet ^r)	Tet ^r , Hyg ^r /pNBV1	0
	<i>E. coli</i> Y1090 [pMC9(Amp ^r Tet ^r)]	Tet ^r , Hyg ^r /pNBV1	0
pSMT3(Hyg ^r)	<i>E. coli</i> XL-1 Blue(Tet ^r)	Tet ^r , Hyg ^r /pSMT3	0
	<i>E. coli</i> Y1090 [pMC9(Amp ^r Tet ^r)]	Tet ^r , Hyg ^r /pSMT3	0
pNBV1(Apr ^r Hyg ^r) + R6K(Amp ^r Str ^r)	<i>E. coli</i> XL-1 Blue(Tet ^r)	Tet ^r , Hyg ^r /pNBV1	0
	<i>E. coli</i> Y1090 [pMC9(Amp ^r Tet ^r)]	Tet ^r , Hyg ^r /pNBV1	0
	<i>M. bovis</i> BCG wild-type	Pza ^r , Hyg ^r /pNBV1	0
pSMT3(Hyg ^r) + R6K(Amp ^r Str ^r)	<i>E. coli</i> XL-1 Blue(Tet ^r)	Tet ^r , Hyg ^r /pNBV1	0
	<i>E. coli</i> Y1090 [pMC9(Amp ^r Tet ^r)]	Tet ^r , Hyg ^r /pNBV1	0
	<i>M. bovis</i> BCG wild-type	Pza ^r , Hyg ^r /pNBV1	0
<i>E. coli</i> S17			
pVK173T(Amp ^r Apr ^r Hyg ^r)	<i>M. smegmatis</i> [pGB9.2(Kan ^r)]	Kan ^r , Apr ^r , Hyg ^r /pVK173T	8.5
	<i>M. tuberculosis gln</i> A1 :: Kan ^r	Kan ^r , Apr ^r , Hyg ^r /pVK173T	1.3
pLC28(Cam ^r Str ^r)	<i>E. coli</i> SM10(Cam ^r Str ^r)	Str ^r , Cam ^r /pLC28	9.5

DISCUSSION

This laboratory's approach to the design of vaccines against tuberculosis and other important mycobacterial infections such as bovine tuberculosis and leprosy is centred on the expression of major mycobacterial extracellular proteins in native form in a heterologous mycobacterial vaccine host. Our demonstration that a recombinant BCG vaccine overexpressing the *M. tuberculosis* 30 kDa protein is more protective than conventional BCG in the highly relevant guinea pig model of pulmonary tuberculosis has provided impetus for this approach (Horwitz *et al.*, 2000; Horwitz & Harth, 2003). With this study, we are exploring the possibility that expression of multiple mycobacterial proteins in a recombinant BCG strain will yield a vaccine that is even more potent against tuberculosis than a vaccine expressing a single protein. Our approach was to add a second *E. coli*/mycobacteria shuttle vector, plasmid pGB9.2, to the established vectors pSMT3 and pNBV1, because these plasmids are compatible and this strategy may overcome certain limitations of a one-plasmid approach.

Used as single plasmids, the high-copy-number plasmids pNBV1 and pSMT3 express mycobacterial proteins at many multiples of their endogenous level in the wild-type host. In contrast, pGB9.2 is a low-copy-number plasmid, allowing expression of heterologous proteins at low levels. Such low-level expression may more closely resemble the level of the protein in the native mycobacterial host, where the gene encoding the extracellular protein is present as a single copy in the bacterial chromosome.

In a two-plasmid approach, pGB9.2 can be added as a second plasmid to a recombinant BCG bacterium carrying either pNBV1 or pSMT3. This strategy may have several advantages. Firstly, where overexpression of multiple proteins is involved, it decreases the stress on the primary plasmid, which otherwise would be forced to maintain two, three, four or more *M. tuberculosis* genes. Adding additional genes to the same plasmid typically increases the likelihood of a recombination event and the likelihood that plasmid copy number will decrease (G. Harth & M. A. Horwitz, unpublished results). Secondly, the availability of a system involving a low-copy-number plasmid in combination with a high-copy-number plasmid provides the flexibility of expressing some recombinant proteins at low levels and others at high levels. As live recombinant mycobacterial vaccines become more complex and involve the expression of multiple heterologous proteins, optimizing their immunoprotective capacity may require modulating the expression of certain proteins relative to others. Too high an expression of all recombinant proteins may interfere with the immune response to one of the proteins, including endogenous proteins of the recombinant BCG host, or result in other untoward effects, such as the induction of tolerance or a Th2 type of immune response. Thirdly, transcriptional regulation of gene expression is more readily accomplished if the copy number of a target sequence such as a promoter element is low, because the

target can be more readily saturated with binding factors, which otherwise might be diluted out were there too many target copies present in a cell.

The utility of pGB9.2 might be enhanced by further investigation of several aspects of the plasmid. Firstly, even though we trimmed down the plasmid to 11.4 kb from its original size of 14.8 kb, the mycobacterial portion remains largely unmapped. It would be helpful to know if further deletions could be introduced without compromising useful features such as self-transmissibility and plasmid stability in the absence of selective pressure. Secondly, while plasmid stability in the absence of selective pressure was demonstrated *in vitro*, it would be important to confirm plasmid stability *in vivo* in an infection model, such as guinea pigs, were this two-plasmid approach ever found to be superior to our current one-plasmid recombinant BCG approach, and thus worth consideration as an improved version of BCG. Thirdly, although intended for the study of regulatory proteins, several groups of investigators have also developed two-plasmid systems or used mycobacterial plasmids of different compatibility groups to develop additional *E. coli*-mycobacteria shuttle vectors (Picardeau & Vincent, 1997; Gavigan *et al.*, 1997; Kaps *et al.*, 2001; Pashley *et al.*, 2003). Presumably, our system would also be appropriate for such studies and merits investigation in this regard.

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