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.

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
transf erase. 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 cam′ gene, a partial tet′ gene and part of the intergenic region of pACYC184 between the tet′ gene and the replication origin, ori p15A. The altered version of pGB9 was designated pGB9.2 (Fig. 1). Another deletion eliminated the two PlacZ and lacZs-carrying Asel–Asel 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 Psl site of pGB9.2 (Fig. 1).

Modifications of plasmid pNBV1 for conjugation assays. The mycobacterial shuttle vector pNBV1(Hyg′) was modified by inserting the gene encoding apramycin resistance between the plasmid’s two DruI sites to yield plasmid pNBV1(Apr′Hyg′).

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 mm acetate filters, then concentrated in Amicon Diaflo filtration units to a final concentration of 1×106 cell equivalents ml−1 and assayed 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-harbouring 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.
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: PaeI, SpeI, SwaI, EcoRV and NdeI. 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.
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.
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, 11 of bacterial culture (~5 × 10^9 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Endogenous 30 kDa protein</th>
<th>Endogenous + recombinant 30 kDa protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. smegmatis</em></td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td><em>M. bovis BCG</em></td>
<td>0.5</td>
<td>2.8</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>1.1</td>
<td>6.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*30 kDa protein (FbpB; mycolyl transferase) in *M. smegmatis* ~27 kDa; in BCG and *M. tuberculosis* ~30 kDa.
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 $(7\pm0.8\pm0.2)\times10^7$ c.f.u. ml$^{-1}$ and on kanamycin-lacking medium $(7\pm0.8\pm0.2)\times10^7$ c.f.u. ml$^{-1}$; the corresponding numbers for M. smegmatis were $(5\pm0.8\pm0.2)\times10^6$ c.f.u. ml$^{-1}$ and $(5\pm0.8\pm0.2)\times10^6$ 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 $\approx5\times10^9$ c.f.u., we isolated $\approx20\mu g$ of DNA which contained $\approx70\mu g$ of pGB9.2, as determined by gel scans. Since one pGB9.2 plasmid equals $\approx1.2\times10^{-8}$ ng DNA, we expected $\approx60\mu g$ pGB9.2 DNA in $5\times10^9$ 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 DH5α yielded kanamycin-resistant E. coli bacteria at mean frequencies of $\approx10^6$ transformants per $\mu g$ pGB9.2 plasmid DNA and 5000 transformants per $\mu g$ total bacterial DNA. Using the values mentioned above, we expected $1\mu g$ total DNA to contain $\approx3$ ng pGB9.2 DNA, which would result in $\approx3000$ 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 ($\approx10$ kb), the results of which demonstrated that recombinant BCG bacteria harbouring pMTB30 contain $\approx13$–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 HindIII 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 HindIII and XbaI 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'), we performed a series of bacterial conjugations and compared its mobility with that of pNBV1(Apr'Hyg') and pSMT3(Hyg') (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-thiopere 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 pGB9.2 with either *M. bovis* BCG Tice[pSMT3(Hyg')] or *M. tuberculosis* Erdman[pNBV1(Apr'Hyg')] 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

<table>
<thead>
<tr>
<th>Donors</th>
<th>Recipients</th>
<th>Selection (markers/plasmid)</th>
<th>10⁷ × Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. smegmatis</em> 1-2c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGB9.2(Kan')</td>
<td><em>M. tuberculosis</em> wild-type</td>
<td>TCH’, Kan’/pGB9.2</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td><em>M. bovis</em> BCG [pSMT3(Hyg’)]</td>
<td>Hyg’, Pza’, Kan’/pGB9.2</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em> [pNBV1(Apr’Hyg’)]</td>
<td>Apr’, Hyg’, TCH’, Kan’/pGB9.2</td>
<td>1.8</td>
</tr>
<tr>
<td>pNBV1(Apr’Hyg’)</td>
<td><em>M. tuberculosis</em> gln A1:: Km’</td>
<td>Kan’, TCH’, Apr’, Hyg/pNBV1</td>
<td>0</td>
</tr>
<tr>
<td>pSMT3(Hyg’)</td>
<td><em>M. tuberculosis</em> gln A1:: Km’</td>
<td>Kan’, TCH’, Hyg/pSMT3</td>
<td>0</td>
</tr>
<tr>
<td>pGB9.2(Kan’)+pSMT3(Hyg’)</td>
<td><em>M. tuberculosis</em> wild-type</td>
<td>TCH’, Kan’/pGB9.2</td>
<td>2.3</td>
</tr>
<tr>
<td>pVK173T(Amp’Apr’Hyg’)</td>
<td><em>M. bovis</em> BCG wild-type</td>
<td>Apr’, Hyg’/pVK173T</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em> wild-type</td>
<td>Apr’, Hyg’/pVK173T</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGB9.2(Kan’)</td>
<td><em>E. coli</em> XL-1 Blue(Tet’)</td>
<td>Tet’, Kan’/pGB9.2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> Y1090 [pMC9(Amp’Tet’)]</td>
<td>Tet’, Kan’/pGB9.2</td>
<td>0</td>
</tr>
<tr>
<td>pNBV1(Apr’Hyg’)</td>
<td><em>E. coli</em> XL-1 Blue(Tet’)</td>
<td>Tet’, Hyg/pNBV1</td>
<td>0</td>
</tr>
<tr>
<td>pSMT3(Hyg’)</td>
<td><em>E. coli</em> Y1090 [pMC9(Amp’Tet’)]</td>
<td>Tet’, Hyg/pSMT3</td>
<td>0</td>
</tr>
<tr>
<td>pNBV1(Apr’Hyg’)+R6K(Amp’Str’)</td>
<td><em>E. coli</em> XL-1 Blue(Tet’)</td>
<td>Tet’, Hyg/pNBV1</td>
<td>0</td>
</tr>
<tr>
<td>pSMT3(Hyg’)+R6K(Amp’Str’)</td>
<td><em>E. coli</em> Y1090 [pMC9(Amp’Tet’)]</td>
<td>Tet’, Hyg/pSMT3</td>
<td>0</td>
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<tr>
<td><em>E. coli</em> S17</td>
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<td></td>
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<tr>
<td>pVK173T(Amp’Apr’Hyg’)</td>
<td><em>M. smegmatis</em> [pGB9.2(Kan’)]</td>
<td>Kan’, Apr’, Hyg’/pVK173T</td>
<td>8.5</td>
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<tr>
<td></td>
<td><em>M. tuberculosis</em> glnA1:: Kan’</td>
<td>Kan’, Apr’, Hyg’/pVK173T</td>
<td>1.3</td>
</tr>
<tr>
<td>pLC28(Cam’Str’)</td>
<td><em>E. coli</em> SM10(Cam’Str’)]</td>
<td>Str’, Cam’/pLC28</td>
<td>9.5</td>
</tr>
</tbody>
</table>
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 pMT3 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 pMT3 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 pMT3. 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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REFERENCES


