Transformation of mycobacterial species using hygromycin resistance as selectable marker

Thomas R. Garbe,‡ Jaya Barathi, Simona Barnini, Ying Zhang, Christiane Abou-Zeid, Dan Tang, Rama Mukherjee and Douglas B. Young

INTRODUCTION

The recent development of gene transfer systems for mycobacteria has provided an important new tool for analysis of mechanisms of drug resistance and for generation of novel recombinant vaccines (Aldovini & Young, 1991; Stover et al., 1991; Zhang et al., 1992). The most commonly used systems employ plasmid vectors carrying the origin of replication from a naturally occurring mycobacterial plasmid, pAL5000 (Labidi et al., 1985, 1992), or the chromosomal attachment site from mycobacteriophage L5 (Lee et al., 1991), along with a gene conferring resistance to kanamycin as selectable marker (Snapper et al., 1988; Ranes et al., 1990; Aldovini & Young, 1991; Stover et al., 1991). Radford & Hodgson (1991) have also reported that hygromycin resistance can be used as a selectable marker in transformation experiments with Mycobacterium smegmatis and Mycobacterium bovis BCG. Most work on mycobacterial transformation has focused on the vaccine strain, M. bovis BCG, and on M. smegmatis, a rapid growing non-pathogenic mycobacterium. Although the wild-type M. smegmatis strain can be transformed with only low efficiency [<10 transformants (µg DNA)⁻¹], it has been shown that transformants that have been cured of a plasmid are subsequently able to act as recipients with a relatively high efficiency of transformation [>10⁴ transformants (µg DNA)⁻¹] (Snapper et al., 1990).

We were interested in transforming additional mycobacterial species. In particular, we wished to explore the possibility of using Mycobacterium w (Talwar, 1978) and Mycobacterium vaccae (Stanford et al., 1990) as potential recombinant vaccines. Both have been shown to be safe for humans when used as killed vaccine preparations (Talwar et al., 1990; Stanford et al., 1990), and the potent adjuvant properties of mycobacteria make them attractive candidate vectors for delivery of recombinant antigens from pathogenic mycobacteria or other infectious agents. In addition, we have previously demonstrated advantages associated with the use of M. smegmatis as host for expression of recombinant genes encoding mycobacterial antigens and enzymes (Garbe et al., 1993; Zhang et al., 1991), and we were interested in exploring the potential for using other non-pathogenic mycobacteria in a similar role.

In initial experiments we were unable to demonstrate transformation of these mycobacterial species using previously described vectors employing kanamycin re-
Fig. 1. Vector construction. p16R1 was generated by insertion of a 2.6 kb fragment derived from pYUB12 and containing the origin of replication from mycobacterial plasmid pAL5000 ('ALori') into the unique BamHI site in pIJ963. Analysis of the resulting plasmid revealed a deletion of a 1.9 kb fragment including the AmpR region of pIJ963. pBSH5 was generated by insertion of a 1.75 kb fragment containing HygR from plJ963 into the BamHI site in pYZ5. The resulting plasmid was unable to replicate in E. coli, and is assumed to have undergone deletion and/or rearrangement.

sistance as a selectable marker. In this report we describe the general utility of hygromycin resistance as a selectable marker for several mycobacterial strains, and demonstrate the application of a novel hygromycin resistance vector for overexpression of the superoxide dismutase enzyme from Mycobacterium tuberculosis.

METHODS

Bacterial cultures and electroporation. For electroporation experiments, all of the mycobacteria were grown in Middlebrook 7H9 medium supplemented with albumin, dextrose and catalase (ADC), according to the manufacturer’s recommendations (Difco). Mycobacterium w (Talwar, 1978) was grown as standing cultures at 37 °C; M. vaccae (kindly supplied by Dr John Stanford, University College and Middlesex Hospital School of Medicine, London) was grown with shaking at 30 °C; M. smegmatis (a derivative of strain mc26 selected for improved transformation efficiency) (Zhang et al., 1991) and M. bovis BCG (Glaxo strain) were grown in shaking cultures at 37 °C.

For electroporation experiments, cultures (200 ml) with an A600 of 0.5–1.0 were processed as described previously by chilling on ice and washing with glycerol (Zhang et al., 1991). Bacterial suspensions in glycerol (0–1–0.4 ml) were mixed with plasmid DNA (in 1–5 μl deionized water) and subjected to electroporation using a Bio-Rad Gene Pulser as previously described (Zhang et al., 1991). Cold 7H9/ADC medium (0.4–0.5 ml) was added and, after incubation for 2–4 h to allow phenotypic expression, aliquots (0.1–0.2 ml) were spread on agar plates prepared using Middlebrook 7H11 medium supplemented with oleic acid, albumin, dextrose, catalase (OADC; Difco) and appropriate antibiotics. Kanamycin sulphate (Sigma) was used at a concentration of 50 μg ml⁻¹. Hygromycin B (Sigma) was supplied in solution (460000 units ml⁻¹, with one unit corresponding to 455 ng) and was used at 50 μg ml⁻¹ for mycobacterial cultures, and 200 μg ml⁻¹ for Escherichia coli. Plates were incubated at 37 °C (30 °C for M. vaccae) with transformed colonies emerging after 3–5 d (M. smegmatis and M. vaccae) and 10–14 d (Mycobacterium w) and 3–4 weeks (M. bovis BCG).

Plasmids and vector construction. The Mycobacterium–Escherichia coli shuttle vector, pBAK14, has been described previously, along with pYZ5, a pBAK14 derivative containing the M. tuberculosis sodA gene on a 1.1 kb EcoRI–KpnI fragment (Zhang et al., 1991). Two additional mycobacterial shuttle vectors – pYUB12 (Snapper et al., 1988) and pMV251 (Stover et al., 1991) – were kindly provided by Dr Bill Jacobs (Albert Einstein College of Medicine, New York, USA) and Dr Ken Stover (MedImmune, Gaithersburg, Va, USA), respectively. pIJ963 (provided by Dr Tobias Kieser, John Innes Institute, Norwich, UK) is a derivative of pUC18 carrying the hygromycin
The characteristics of plasmids used in this study are listed in Table 1.

### Table 1. Characteristics of plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
<th>Drug selection</th>
<th>Ability to transform different host strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>pBAK14</td>
<td>Zhang et al. (1991)</td>
<td>Kanamycin</td>
<td>Yes</td>
</tr>
<tr>
<td>pYUB12</td>
<td>Snapper et al. (1988)</td>
<td>Kanamycin</td>
<td>Yes</td>
</tr>
<tr>
<td>pMV251</td>
<td>Stover et al. (1991)</td>
<td>Kanamycin</td>
<td>Yes</td>
</tr>
<tr>
<td>pIJ963</td>
<td>Lydiate et al. (1989)</td>
<td>Hygromycin</td>
<td>Yes</td>
</tr>
<tr>
<td>pAL5000</td>
<td>(natural plasmid from M. fortuitum)</td>
<td>None</td>
<td>NT</td>
</tr>
<tr>
<td>p16R1*</td>
<td>pIJ963 + origin of replication from pAL5000</td>
<td>Hygromycin</td>
<td>Yes</td>
</tr>
<tr>
<td>pYZ5</td>
<td>pBAK14 + indA (Zhang et al., 1991)</td>
<td>Kanamycin</td>
<td>Yes</td>
</tr>
<tr>
<td>pBSH5*</td>
<td>pYZ5 + bg from pIJ963</td>
<td>Hygromycin</td>
<td>No</td>
</tr>
</tbody>
</table>

NT, Not tested.

* Deletion/rearrangement occurred during vector construction.

### Table 2. Transformation frequencies for different mycobacterial species

Bacterial cells were transformed by electroporation with pBAK14 or p16R1 and aliquots were plated out in the presence of kanamycin or hygromycin, respectively. Results are expressed as the mean number of colonies on duplicate samples from a single representative experiment. The efficiency of transformation varied by as much as 10-fold between different batches of competent cells, but the differential efficiency of the two vectors was observed in all experiments. NT, Not tested.

<table>
<thead>
<tr>
<th>DNA concn (µg)</th>
<th>M. smegmatis</th>
<th>M. bovis BCG</th>
<th>M. vaccae</th>
<th>Mycobacterium w</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pBAK14</td>
<td>p16R1</td>
<td>pBAK14</td>
<td>p16R1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>11</td>
<td>6 x 10⁴</td>
<td>1</td>
<td>2 x 10³</td>
</tr>
<tr>
<td>1.25</td>
<td>2 x 10⁵</td>
<td>5 x 10³</td>
<td>1 x 10⁴</td>
<td>8 x 10³</td>
</tr>
<tr>
<td>2</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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</tr>
<tr>
<td>5</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

The characteristics of plasmids used in this study are listed in Table 1.

### Analysis of expressed proteins

For analysis of protein expression, M. vaccae and M. smegmatis were grown in Middlebrook 7H9 medium supplemented with glucose (2%, w/v); for Mycobacterium w, 7H9 medium was supplemented with ADC. Protein expression in transformants was analysed by PAGE of extracts prepared by glass-bead disruption of bacterial cells (Zhang et al., 1991). For SDS-PAGE samples (10–20 µg protein) were treated with sample buffer containing SDS and β-mercaptoethanol and separated in discontinuous gels containing 12% (w/v) acrylamide as described by Laemmli (1970). Gels were then stained with Coomassie Brilliant blue or transferred to nitrocellulose membranes for Western blot analysis (Towbin et al., 1979). Blots were then stained with a monoclonal antibody (D2D) against mycobacterial superoxide dismutase as described previously (Zhang et al., 1991). The electrophoretic mobility of recombinant superoxide dismutase enzymes was examined by...
activity staining of gels run under non-denaturing conditions (Zhang et al., 1991).

RESULTS

Construction of plasmid vectors containing the hyg gene

Two strategies were adopted for construction of shuttle vectors containing the hygromycin resistance gene along with a mycobacterial origin of replication. In the first approach, a fragment containing the replication origin of the cryptic mycobacterial plasmid pAL5000 was inserted into the hygromycin-resistance-expressing plasmid pJ963 and the ligation mixture transformed directly into M. smegmatis. Two transformed colonies were examined in detail. From one (16R1) a plasmid conferring hygromycin resistance could be recovered and propagated in E. coli when selected for Hyg'. Further analysis of the plasmid (p16R1) revealed that a 1.9 kb deletion including the AmpR region of the pUC component had occurred (Fig. 1). Plasmid DNA isolated from the second colony (16R2) was capable of retransforming E. coli. We infer that a deletion or a rearrangement had also occurred in this plasmid, and no further characterization was attempted.

The second strategy was to ligate a DNA fragment containing the hyg gene into a previously characterized mycobacterial shuttle plasmid, pYZ5 (Zhang et al., 1991) (Fig. 1). In this case, the ligation mixture was used to transform M. vaccae. As had been found for 16R2 above, plasmids isolated from transformants could not be transformed into E. coli (Hyg' selection), although successful retransformation of M. vaccae was readily demonstrated. Again, we infer that alterations in the plasmid had resulted in loss of the E. coli origin of replication.

Transformation efficiency with hyg vectors

The shuttle plasmid p16R1 (hyg) was tested for its ability to transform several mycobacterial species, and compared with pBAK14, a shuttle vector with a kanamycin resistance determinant. In each of the four species tested, the number of transformants obtained after electroporation with p16R1 was greater than that obtained with the same amount of pBAK14 DNA (Table 2). The difference was particularly pronounced in the case of M. vaccae and Mycobacterium w. We were unable to demonstrate transformation in either of these species using kan vectors (pBAK14, pYUB12 and pMV251), whereas the hyg vector consistently yielded $10^2$-$10^3$ transformants ($\mu$g DNA)$^{-1}$. Even with M. bovis BCG and M. smegmatis, which were previously shown to be transformable with kan vectors, transformation frequencies with p16R1 were greater than those observed with pBAK14. We also noted a difference in the rate of growth of the two types of transformants. p16R1 transformants of M. smegmatis emerged on the Hyg plates 1–2 d before pBAK14 transformants appeared on Kan plates. Similarly, a difference of up to 1 week was noted for M. bovis BCG transformants. In each case, drug-resistant colonies were presumed to be genuine transformants since: (a) there was little or no spontaneous drug resistance in controls without DNA; (b) each time an individual resistant colony was tested, it was found to express the expected recombinant protein (see below).

Expression of M. tuberculosis superoxide dismutase in different mycobacteria

We then investigated the use of hyg vectors for expression of a heterologous protein in different mycobacterial species. Superoxide dismutase from M. tuberculosis was selected as a 'model' protein for these experiments. We have previously demonstrated the introduction of the recombinant sodA gene into M. smegmatis where, in contrast to E. coli, it is expressed in a functionally active form (Zhang et al., 1991). pBSH5, generated by introduction of the hyg gene into a shuttle vector already

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Fig. 2. Expression of M. tuberculosis sodA in recombinant mycobacteria. Mycobacterial extracts were analysed by SDS-PAGE and stained for total protein (a), or for mycobacterial superoxide dismutase by Western blot with monoclonal antibody D2D (b). Migration positions of standard molecular mass markers are indicated on the left of the figure. Lanes: 1, M. tuberculosis; 2, M. vaccae(p16R1); 3, M. vaccae(pBSH5); 4, Mycobacterium w(p16R1); 5, Mycobacterium w(pBSH5). The recombinant M. tuberculosis superoxide dismutase is seen as a major band in lanes 3 and 5 (a, Mtb). The native superoxide dismutase from M. vaccae reacted only weakly with D2D (b, lane 2). Mycobacterium w superoxide dismutase (Mw) was recognized by D2D and has a higher apparent molecular mass than the M. tuberculosis protein (Mtb) (b, lanes 4 and 5).
Fig. 3. Characterization of recombinant superoxide dismutase enzymes. Mycobacterial extracts were stained for superoxide dismutase activity after fractionation by non-denaturing PAGE. Lanes: 1, *M. tuberculosis*; 2, *M. vaccae* (p16R1); 3, *M. vaccae* (pBSH5); 4, *M. smegmatis* (p16R1); 5, *M. smegmatis* (pBSH5). Expression of *M. vaccae* sodA gene (Fig. 1), was used to transform *M. vaccae* and *Mycobacterium w*. Transformants were analysed by SDS-PAGE, and sodA expression was visualized by Coomassie Brilliant blue and specific antibody staining (Fig. 2). In each case transformants expressed the sodA gene at a high level. Similar results were obtained following transformation with a plasmid constructed by insertion of the sodA gene into the unique KpnI site of p16R1. Densitometric scanning of SDS-PAGE gels indicated that the 23 kDa band corresponding to the recombinant protein accounted for more than 10% of the total protein in extracts from transformed cells. Examination of the migration of the recombinant enzyme during non-denaturing PAGE demonstrated an intriguing difference between the recombinant enzyme expressed in *M. vaccae* and that previously reported in *M. smegmatis* (Fig. 3). Expression of *M. tuberculosis* sodA in *M. smegmatis* results in the generation of five presumably hybrid tetrameric enzymes formed by combination of subunits between the host and the plasmid-specified superoxide dismutases. In contrast, in *M. vaccae*, the predominant superoxide dismutase band in the recombinant strain has a migration pattern identical to that of the native *M. tuberculosis* enzyme. The pattern of recombinant superoxide dismutase expression in *Mycobacterium w* resembled that seen in *M. smegmatis*.

**DISCUSSION**

Our results indicate that selection on the basis of hygromycin resistance may have certain advantages over the use of kanamycin resistance in mycobacterial transformation experiments. This is particularly true in experiments using mycobacterial species other than laboratory strains of *M. smegmatis* and *M. bovis* BCG. We have previously reported the difficulties in transforming clinical isolates of *M. tuberculosis* (Zhang et al., 1992) and we have recently been able to overcome these difficulties using the p16R1 vector (Zhang et al., 1993). The reason for the improved transformation frequency offered by hygromycin selection has not been elucidated. The fact that two different hyg vectors (p16R1 and pBSH5) can transform *M. vaccae* and *Mycobacterium w*, whilst three kan vectors (pBAK14, pYUB12 and pMV251) were unsuccessful, tends to suggest that it is the drug selection itself which is important, rather than some other property of the particular vector. The hyg resistance gene from *Streptomyces hygroscopicus* may be more efficiently expressed in mycobacteria than the E. coli-derived aminoglycoside phosphotransferase gene involved in kanamycin resistance. However, it is important to note that the hyg vectors were prepared by electroporation of ligation mixtures directly into mycobacterial recipients, and it is possible that subsequent recombination events may have conferred enhanced stability of these plasmids in mycobacterial hosts. In addition to broadening the potential host range for mycobacterial transformation, hygromycin resistance is attractive in providing a second selectable marker for genetic experiments in mycobacteria, and in providing a resistance marker which avoids use of a clinically applicable drug.

*M. smegmatis* is a useful model system for mycobacterial genetic experiments because of its ease of laboratory manipulation in terms of lack of pathogenicity and relatively rapid growth rate. However, other mycobacterial species may provide more useful models for studying some specific aspects of mycobacterial physiology or biochemistry. Thus we have demonstrated a role for *M. vaccae* as a host for overexpression of *M. tuberculosis* superoxide dismutase. Whilst transformation with *M. tuberculosis* sodA generates hybrid enzymes in *M. smegmatis* (Zhang et al., 1991), the *M. vaccae* expression system allows us to obtain the recombinant superoxide dismutase apparently in the native form. This system is currently being used to produce large amounts of the enzyme (10 mg purified protein per litre of culture) for structural studies (Cooper et al., 1994).

*Mycobacterium w* has been administered as an immunotherapeutic agent to patients undergoing treatment for lepromatous leprosy and is currently undergoing clinical trials as a prophylactic anti-leprosy vaccine (Talwar et al., 1990; Zaheer et al., 1993). *M. vaccae* has been used as a vaccine in a series of human trials and is currently being tested for efficacy in immunotherapy of tuberculosis (Stanford et al., 1990). In contrast to *M. bovis* BCG, both of these mycobacteria are administered as killed vaccine preparations. The known adjuvant properties of mycobacteria make it attractive to suggest that *Mycobacterium w* and *M. vaccae* could be used as carriers to deliver heterologous antigens to the immune system; either by mixing purified antigens with existing vaccines, or by taking advantage of recombinant DNA techniques to achieve expression of the antigens in mycobacterial cells. The techniques developed in this study provide a means for pursuing such an objective.
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REFERENCES


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