Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*

Ellen M. Foley-Thomas,1 Diana L. Whipple,2 Luiz E. Bermudez3 and Raúl G. Barletta1

Author for correspondence: Raúl G. Barletta. Tel: +1 402 472 8543. Fax: +1 402 472 9690.

*Mycobacterium avium* complex strains and *Mycobacterium paratuberculosis* are closely related intracellular pathogens affecting humans and animals. *M. avium* complex infections are a leading cause of morbidity and mortality in AIDS patients, and *M. paratuberculosis* is the agent of Johne's disease in ruminants. Genetic manipulation of these micro-organisms would facilitate the understanding of their pathogenesis, the construction of attenuated vaccine strains and the development of new drugs and treatment methods. This paper describes the replication of mycobacterial shuttle plasmids and plasmids, and the expression of the firefly luciferase reporter gene in *M. avium* complex and *M. paratuberculosis*. The mycobacteriophage TM4 propagated on *M. smegmatis* or *M. paratuberculosis* plaqued at the same efficiency on these two mycobacterial hosts. Screening of *M. avium* complex and *M. paratuberculosis* clinical isolates with TM4-derived luciferase reporter phages demonstrated that the majority of these isolates were susceptible to TM4. Conditions for introduction of DNA were determined by transfection of *M. paratuberculosis* with TM4 DNA and applied to isolate kanamycin-resistant transformants of *M. avium* complex and *M. paratuberculosis* with *Escherichia coli--Mycobacterium shuttle plasmids*. Recombinant plasmids were recovered from transformants without apparent loss of DNA sequences. These results provide the basis for the genetic manipulation of these pathogenic mycobacterial species.

**Keywords:** *Mycobacterium avium* complex, *Mycobacterium paratuberculosis*, mycobacteriophage, shuttle vectors, transformation

INTRODUCTION

*Mycobacterium avium* complex strains and *Mycobacterium paratuberculosis* are facultative intracellular pathogens able to replicate in mononuclear phagocytes. The *M. avium* complex includes 28 serovars of two species, *M. avium* and *M. intracellulare*. *M. avium* complex infections are a leading cause of morbidity and mortality for AIDS patients. These patients tend to develop bacteraemia and disseminated infections later in the course of the disease (Hawkins et al., 1986; Inderlied et al., 1993; Young, 1988). The prevalence of *M. avium* complex infections of the lung has also increased in patients with predisposing conditions such as bronchogenic carcinoma and chronic obstructive lung disease associated with smoking (Horsburgh et al., 1985). *M. paratuberculosis* is the causative agent of paratuberculosis or Johne's disease in ruminants (Chiodini et al., 1984). Recent isolation of *M. paratuberculosis* from patients affected with Crohn's disease suggests a potential pathogenic role in humans (McFadden et al., 1987). Paratuberculosis in ruminants is characterized by diarrhoea and weight loss associated with progressive granulomatous enteritis (Chiodini et al., 1984).

*M. avium* and *M. paratuberculosis* are slow-growing mycobacteria. DNA–DNA hybridization studies have shown that these micro-organisms belong to a single genomic species (Hurley et al., 1988), and it has been proposed to reclassify *M. paratuberculosis* as a subspecies of *M. avium* (Thorel et al., 1990). Furthermore, all *M. paratuberculosis* strains are characterized by the presence of the insertion sequence IS900 (Green et al., 1989), which is absent from *M. avium* strains. Phenotypic differences between *M. avium* and *M. paratuberculosis*, such as mycobactin requirement, ability to grow on egg medium,
growth stimulation by pyruvate, and tolerance to cycloserine correlate with variations in pathogenicity and host range (Thorel et al., 1990).

The development of genetic tools to manipulate mycobacteria has proceeded rapidly (Jacobs et al., 1991). However, studies with M. avium complex and M. paratuberculosis have lagged behind those with M. smegmatis, M. tuberculosis and M. bovis BCG. In this study, phage infection, transfection and transformation of M. avium complex and M. paratuberculosis were examined. Infection of prototype strains and clinical isolates with wild-type mycobacteriophages and reporter shuttle plasmids is demonstrated. We also report the replication of shuttle plasmids derived from the M. fortuitum plasmid pAL5000 (Labidi et al., 1984; Snappet et al., 1988; Stover et al., 1991), and the isolation of plasmid transfectants expressing the firefly luciferase gene. These tools should make possible the genetic manipulation of these microorganisms for the construction of attenuated vaccine strains, the study of the mechanisms of pathogenesis, and the development of new antibiotics and other treatment methods.

METHODS

Bacterial strains, phages and plasmids. Escherichia coli and mycobacterial strains, mycobacteriophages and shuttle plasmids are described in Table 1. M. paratuberculosis strains K-10 and S-23 were bovine isolates with two to three in vitro passages and confirmed to be M. paratuberculosis by a DNA probe detection assay (Idexx Laboratories) and their characteristic mycobactin dependency (Thorel et al., 1990). M. smegmatis cultures were grown with shaking at 37 °C in Middlebrook 7H9 broth supplemented with albumin/dextrose complex and 0.05% Tween 80. M. paratuberculosis and M. avium complex cultures were grown standing at 37 °C in Middlebrook 7H9 broth, adjusted to pH 5.9, and supplemented with oleic acid/albumin/dextrose complex and 0.05% Tween 80. Ferric mycobactin J (Allied Monitor) at 1.0 µg ml⁻¹ was added for M. paratuberculosis cultures. For solid media, Tween was omitted and Bacto Agar was added to 7H9 Middlebrook medium at 0.05% concentration for selection of plasmid transformants, approximately 10⁷ c.f.u. ml⁻¹.

Mycobacteriophage infection assays. Phage lysates (0.1 ml of duplicate 10-fold serial dilutions) were incubated with 0.2 ml of fresh mycobacterial cultures, corresponding to approximately 3.0 x 10⁹ c.f.u., for 30 min at room temperature. Middlebrook 7H9 soft agar (0.7%) was added, and the cells were plated on 7H9 Middlebrook medium by the soft agar layer method as described by Adams (1959) and incubated at 37 °C until either plaques or confluent lawns developed (1 to 3 d for M. smegmatis, 2 to 4 weeks for M. avium complex strains and M. paratuberculosis). Phage titres were determined at dilutions that gave single isolated plaques to exclude the possibility of lysis from without.

Infection with luciferase reporter plasmids and luciferase assays. Infection with luciferase reporter phages and luciferase assays were performed as described by Jacobs et al. (1993). Mycobacterial cultures were grown to exponential phase (OD₆₅₀ 0.2; approximately 6.0 x 10⁸ c.f.u. ml⁻¹) in Middlebrook 7H9 medium, washed three times in growth medium without Tween, diluted fivefold in fresh medium and grown in standing conditions at 37 °C for 48 h to an OD₆₅₀ of approximately 0.1. From these cultures 1.0 ml (approx. 3.0 x 10⁹ c.f.u.) was infected with 1.0 x 10⁹ p.f.u. of phAE39 or phAE40. Duplicate 0.1 ml samples were withdrawn and diluted with 0.25 ml Middlebrook 7H9 broth without Tween. The luciferase activity was measured at 0, 30, 60, 120 and 240 min using a luminometer (EG&G Berthold AutoLumat LB953) after automated injection of 0.1 ml 1.0 mM luciferin in 0.45 M sodium citrate buffer.

Transfection and transformation of mycobacteria. Conditions for transfection and transformation of M. avium complex and M. paratuberculosis were optimized based on previous studies with M. smegmatis and BCG (Barletta et al., 1992; Jacobs et al., 1991; Lugosi et al., 1989; Snapper et al., 1988, 1990). Mycobacterial cells were grown in complete Middlebrook 7H9 medium as described above. After the culture reached the desired density, cells were incubated on ice for 3 h, harvested, washed and resuspended in 10% (v/v) glycerol at an approximate cell density of 1.0 x 10⁸ c.f.u. ml⁻¹. The concentrated cell suspensions (0.4 ml) were mixed with phage (5.0 µg) or plasmid (1.0 µg). DNA, and electroporated (BioRad Gene Pulser, 2500 V, 1000 µF, 0.2 cm-gap cuvettes). Immediately after electroporation, cells were diluted in Middlebrook 7H9 medium, and incubated for 2 h at room temperature. For transfection experiments, the electroporated cells were mixed with approximately 5.0 x 10⁵ c.f.u. fresh cells, plated on Middlebrook 7H9 medium by the soft agar layer method as described for phage infection assays, and incubated at 37 °C. Cells transformed with plasmid DNA were plated on Middlebrook 7H9 agar supplemented with 50 µg kanamycin ml⁻¹ and incubated at 37 °C. Cells transformed with plasmid DNA were plated on Middlebrook 7H9 agar supplemented with 50 µg kanamycin ml⁻¹ and incubated at 37 °C.

Antibiotic susceptibility assays. To determine the minimum concentration of kanamycin necessary for selection of M. avium complex and M. paratuberculosis transformants, approximately 5.0 x 10⁸ c.f.u. were plated on complete Middlebrook 7H9 agar plates containing either 10, 25, 50 or 100 µg kanamycin ml⁻¹. Susceptibilities of M. avium complex transformants and host strains to kanamycin and amikacin were determined using BACTEC 12B medium (Becton Dickinson) by a radiometric assay as previously described (Inderiedi et al., 1987).

RESULTS

Phage susceptibility studies

Mycobacteriophage infection assays were first used to evaluate transfer of genetic material into M. avium complex and M. paratuberculosis. Although plasmids have been observed in M. avium–intracellulare–iscrofumaeus complex (Crawford & Falkingham, 1990; Martín et al., 1990), no naturally occurring plasmids have been reported in M. paratuberculosis. Another limitation with the use of plasmids was the lack of knowledge about markers to...
Table 1. Bacterial strains, mycobacteriophages and plasmids

<table>
<thead>
<tr>
<th>Strain, phage or plasmid</th>
<th>Source and/or reference and relevant characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Gibco BRL; <em>recA lacZΔM15</em></td>
</tr>
<tr>
<td><em>M. smegmatis</em> mc^155</td>
<td>W. R. Jacobs Jr (Albert Einstein College of Medicine, New York) Snapper <em>et al.</em> (1990) high-efficiency transformation mutant</td>
</tr>
<tr>
<td><em>M. paratuberculosis</em></td>
<td></td>
</tr>
<tr>
<td>ATCC 19698</td>
<td>Merkal (1979)</td>
</tr>
<tr>
<td>K-10</td>
<td>This study</td>
</tr>
<tr>
<td>S-23</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. avium</em> complex</td>
<td></td>
</tr>
<tr>
<td>Strain 18</td>
<td>Sneath &amp; Skerman (1966); Thorel <em>et al.</em> (1990); <em>M. avium</em> serovar 2, ex <em>M. paratuberculosis</em> strain 18</td>
</tr>
<tr>
<td>mc^71</td>
<td>W. R. Jacobs Jr; originally from P. J. Brennan’s collection (Colorado State University); <em>M. avium</em> Bridge, serovar 2</td>
</tr>
<tr>
<td>mc^74</td>
<td>W. R. Jacobs Jr; <em>M. avium</em> 158, serovar 2</td>
</tr>
<tr>
<td>mc^76</td>
<td>W. R. Jacobs Jr; <em>M. avium</em> TMC 1419, serovar 2</td>
</tr>
<tr>
<td>mc^77</td>
<td>W. R. Jacobs Jr; <em>M. avium</em> TMC 1461, serovar 2</td>
</tr>
<tr>
<td>MAC100</td>
<td>Inderlied <em>et al.</em> (1993); isolated from AIDS patient, <em>M. avium</em>, serovar 8</td>
</tr>
<tr>
<td>MAC101</td>
<td>Inderlied <em>et al.</em> (1993); isolated from AIDS patient, <em>M. avium</em>, serovar 1</td>
</tr>
<tr>
<td>MAC109</td>
<td>Inderlied <em>et al.</em> (1993); isolated from AIDS patient, <em>M. avium</em>, serovar 4</td>
</tr>
<tr>
<td>8624-86</td>
<td>R. C. Good (Centers for Disease Control and Prevention, Atlanta); <em>M. intracellulare</em>, serovar 14</td>
</tr>
<tr>
<td>8626-86</td>
<td>R. C. Good; <em>M. intracellulare</em>, serovar 16</td>
</tr>
<tr>
<td>8627-86</td>
<td>R. C. Good; <em>M. intracellulare</em>, serovar 16</td>
</tr>
<tr>
<td><strong>Mycobacteriophages</strong></td>
<td></td>
</tr>
<tr>
<td>D29</td>
<td>Froman <em>et al.</em> (1954)</td>
</tr>
<tr>
<td>ph60</td>
<td>W. R. Jacobs Jr; soil isolate</td>
</tr>
<tr>
<td>ph72</td>
<td>W. R. Jacobs Jr; soil isolate</td>
</tr>
<tr>
<td>phAE39</td>
<td>Jacobs <em>et al.</em> (1993); TM4-derived shuttle plasmid carrying the firefly luciferase gene downstream from the heat shock BCG hsp60 promoter; replicates as a phage in mycobacteria and as a plasmid in <em>E. coli</em>, plaques at low efficiency on BCG</td>
</tr>
<tr>
<td>phAE40</td>
<td>Jacobs <em>et al.</em> (1993); TM4-derived shuttle plasmid carrying the firefly luciferase gene downstream from the heat shock BCG hsp60 promoter; replicates as a phage in mycobacteria and as a plasmid in <em>E. coli</em>, plaques at high efficiency on BCG</td>
</tr>
<tr>
<td>Bxb1</td>
<td>Barletta <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>L1</td>
<td>Snapper <em>et al.</em> (1988)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pMV261</td>
<td>MedImmune (Gaithersburg, MD, USA); Stover <em>et al.</em> (1991); 4.5 kb, kanamycin-resistance, replicates in <em>E. coli</em> and mycobacteria</td>
</tr>
<tr>
<td>pMV262</td>
<td>MedImmune; Connell <em>et al.</em> (1993); 4.5 kb, kanamycin-resistance, replicates in <em>E. coli</em> and mycobacteria</td>
</tr>
<tr>
<td>pYUB180</td>
<td>Jacobs <em>et al.</em> (1993); 6.5 kb, kanamycin-resistance, carries the firefly luciferase gene downstream from pMV261 hsp60 promoter, replicates in <em>E. coli</em> and mycobacteria</td>
</tr>
</tbody>
</table>

select *M. avium* complex and *M. paratuberculosis* transformants carrying plasmid DNA. Conversely, phage susceptibility assays offered simplicity because plaque formation does not require the survival of the mycobacterial host.

*M. paratuberculosis* strains were tested against the group of mycobacteriophages described in Table 1. Given the close relationship between *M. avium* and *M. paratuberculosis*, the broad-host-range mycobacteriophage TM4, originally isolated from a *M. avium* lysogen (Timme & Brennan,
Table 2. Plaquing efficiencies of mycobacteriophages on *M. smegmatis* and *M. paratuberculosis*

Mycobacteriophages were propagated on *M. smegmatis* or *M. paratuberculosis*. Phage titres were determined by counting the number of isolated plaques and taking the mean from two independent experiments ± SD.

<table>
<thead>
<tr>
<th>Phage (propagation host)</th>
<th>Plaques propagated on <em>M. smegmatis</em> mc²155</th>
<th>Phages propagated on <em>M. paratuberculosis</em> (phenotype/propagation host)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM4 mc²155</td>
<td>5 ± 2 ± 0 ± 2 x 10⁹</td>
<td>2.5 ± 0 ± 1 x 10⁷ (K-10)</td>
</tr>
<tr>
<td>D29 ATCC 19698</td>
<td>7 ± 2 ± 0 ± 2 x 10⁹</td>
<td>1.4 ± 0 ± 3 x 10⁷</td>
</tr>
<tr>
<td>ph60 K-10</td>
<td>8 ± 2 ± 0 ± 2 x 10⁹</td>
<td>2.7 ± 0 ± 5 x 10⁷</td>
</tr>
<tr>
<td>ph72 S-23</td>
<td>9 ± 3 ± 0 ± 2 x 10⁹</td>
<td>2.4 ± 0 ± 3 x 10⁷</td>
</tr>
</tbody>
</table>

Fig. 1. Kinetics of light output resulting from infection of *M. smegmatis* mc²155 (●), *M. avium* mc²76 (■) and *M. paratuberculosis* K-10 (▲) with the luciferase reporter phage phAE39. Data indicate the luciferase activities from a representative experiment.

1984), was likely to infect *M. paratuberculosis*. In addition, the better-characterized mycobacteriophages D29 and L1, and the less-characterized soil isolates ph60, ph72 and Bxb1 were tested. Mycobacteriophage stocks were propagated on *M. smegmatis* and plaqued on various *M. paratuberculosis* strains (Table 2). TM4 and phAE39, a shuttle phasmid derived from TM4, plaqued at high efficiency on *M. paratuberculosis* strains ATCC 19698, and isolates K-10 and S-23. Phage ph60 plaqued at reduced and highly variable host-dependent efficiencies, and phages Bxb1, D29, ph72 and L1 did not plaque. Mycobacteriophages TM4, phAE39 and ph60 formed large clear plaques on *M. paratuberculosis*, indicating a lytic infection. Phage TM4 propagated on *M. paratuberculosis* produced titres 1000-fold lower than when propagated on *M. smegmatis*. Since we did not standardize phage propagation on *M. paratuberculosis*, this difference may be explained either by technical reasons or by variations in the phage-host interactions. Nevertheless, TM4 propagated on *M. paratuberculosis* was also able to plaque at the same efficiency on both *M. smegmatis* and *M. paratuberculosis*. Thus, TM4 and TM4-derived shuttle phasmids could provide a system to shuttle genetic material between *M. smegmatis* and *M. paratuberculosis*. We demonstrated this genetic transfer by infecting *M. paratuberculosis* with luciferase reporter phages propagated on *M. smegmatis* (see below).

Selected *M. avium* complex strains were also tested for phage susceptibility using a rapid spot-test assay. Appropriate serial dilutions of phage stocks were spotted onto 7H9 Middlebrook agar plates seeded with *M. avium* complex strains mc²71, mc²74, mc²76 and mc²77. After two weeks incubation at 37°C, areas of phage lysis with isolated plaques were observed with each strain using ph60, TM4 and the TM4-derived phasmids (data not shown). Other phages tested (see Table 1) did not plaque.

Expression of the firefly luciferase gene

Gene fusion technology (Silhavy et al., 1984) has been applied with success to study the regulation of virulence determinants (Dorman et al., 1990; Galán & Curtiss, 1990; Mahan et al., 1993; Miller et al., 1987; Murphy et al.,
**Table 3. Transfection of M. paratuberculosis**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phage DNA</th>
<th>OD_{600} of culture at harvest</th>
<th>Transfection efficiency (p.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. smegmatis mc^155</td>
<td>D29</td>
<td>0.7</td>
<td>396 ± 20</td>
</tr>
<tr>
<td></td>
<td>TM4</td>
<td>0.7</td>
<td>132 ± 12</td>
</tr>
<tr>
<td>M. paratuberculosis K-10</td>
<td>D29</td>
<td>0.5</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>TM4</td>
<td>0.3</td>
<td>16 ± 5</td>
</tr>
<tr>
<td></td>
<td>TM4</td>
<td>0.5</td>
<td>4 ± 2</td>
</tr>
<tr>
<td></td>
<td>TM4</td>
<td>1.0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td>TM4</td>
<td>1.0*</td>
<td>None</td>
</tr>
</tbody>
</table>

*Culture was harvested in stationary phase, after OD_{600} had remained at 1.0 for approximately 1 week.

Transfection efficiencies were determined by counting the number of isolated plaques resulting from transfection with 50 µg of phage DNA and taking the mean from two independent transformations ± SD. Cells incubated in the absence of phage DNA or with phage DNA treated with deoxyribonuclease yielded no plaques.

a light output. The M. avium complex strains 8624-86, 8626-86 and 8627-86 were not tested in this survey.

**Transfection of M. paratuberculosis**

Since mycobacteriophage TM4 readily infected M. paratuberculosis, TM4 DNA was used to test conditions for the introduction of DNA into M. paratuberculosis. DNA from phage D29, unable to plaque on M. paratuberculosis, was also used. Phage DNA was isolated from phages propagated on M. smegmatis and introduced into M. paratuberculosis by electroporation (Table 3). The transfection experiments showed that plaques on M. paratuberculosis were obtained solely with TM4 DNA, as predicted by the phage infection experiments. Although transfection efficiencies were rather poor, a trend was observed. Transfection of M. paratuberculosis resulted in a lower number of plaques than transfection of M. smegmatis under similar conditions. Transfection of M. paratuberculosis was dependent on the growth phase, with cultures in early exponential phase yielding higher efficiencies. No plaques were obtained by transfection of M. paratuberculosis cells prepared from stationary-phase cultures. These results were then applied to develop conditions for plasmid transformation.

**Transformation of M. avium complex and M. paratuberculosis with plasmid DNA**

Several studies with other mycobacterial systems have relied on the use of kanamycin-resistance (Kan^R) as a selectable phenotype. The majority of available vectors carry the aminoglycoside phosphotransferase I (aph) gene from Tn903 (Oka et al., 1981). Since M. avium complex and M. paratuberculosis strains were resistant to the standard kanamycin concentration (10 µg ml⁻¹) used to select for M. smegmatis transformants, higher antibiotic...
Table 4. Transformation of M. paratuberculosis and M. avium complex with plasmid DNA

Transformation efficiencies were determined by counting the number of kanamycin-resistant colonies (selected at 50 μg ml⁻¹ unless indicated otherwise) obtained per μg plasmid DNA and taking the mean from two independent transformations ± sd. Cells incubated in the absence of plasmid DNA or with plasmid DNA treated with deoxyribonuclease yielded no transformants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid DNA</th>
<th>OD₆₀₀ of culture at harvest</th>
<th>Transformation efficiency [c.f.u. (μg DNA)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. smegmatis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mc6155</td>
<td>pYUB180</td>
<td>0.50</td>
<td>2.8 ± 0.2 x 10⁴*</td>
</tr>
<tr>
<td>mc6155</td>
<td>pYUB180</td>
<td>0.50</td>
<td>5.1 ± 0.8 x 10⁴</td>
</tr>
<tr>
<td>M. paratuberculosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 19698</td>
<td>pYUB180</td>
<td>0.20</td>
<td>1.4 ± 0.1 x 10⁴</td>
</tr>
<tr>
<td>K-10</td>
<td>pMV262</td>
<td>0.23</td>
<td>3.2 ± 0.3 x 10⁴</td>
</tr>
<tr>
<td>K-10</td>
<td>pYUB180</td>
<td>0.23</td>
<td>6.5 ± 0.3 x 10⁴</td>
</tr>
<tr>
<td>S-23</td>
<td>pYUB180</td>
<td>0.20</td>
<td>6.3 ± 0.3 x 10⁴</td>
</tr>
<tr>
<td>M. avium complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mc571</td>
<td>pYUB180</td>
<td>0.20</td>
<td>1.6 ± 0.4 x 10⁴</td>
</tr>
<tr>
<td>mc574</td>
<td>pYUB180</td>
<td>0.20</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>mc576</td>
<td>pYUB180</td>
<td>0.20</td>
<td>1.0 ± 1 x 10⁴</td>
</tr>
<tr>
<td>mc577</td>
<td>pYUB180</td>
<td>0.20</td>
<td>None</td>
</tr>
<tr>
<td>8624-86</td>
<td>pMV261</td>
<td>0.24</td>
<td>7.2 ± 2 x 10³</td>
</tr>
<tr>
<td>8626-86</td>
<td>pMV261</td>
<td>0.23</td>
<td>3.4 ± 0.3 x 10³</td>
</tr>
<tr>
<td>8627-86</td>
<td>pMV261</td>
<td>0.21</td>
<td>2.10 ± 0.02 x 10⁴</td>
</tr>
</tbody>
</table>

* Transforms were selected at 10 μg kanamycin ml⁻¹.

Concentrations were tested. Most M. avium complex and M. paratuberculosis strains were sensitive to 50 μg ml⁻¹. Using the E. coli-Mycobacterium shuttle plasmids pMV262 (Stover et al., 1991; Connell et al., 1993) and pYUB180 (Jacobs et al., 1993), the latter carrying the firefly luciferase gene, we were able to isolate kanamycin-resistant transformants from each M. avium serovar 2 strain, except mc577, and from M. paratuberculosis prototype strain and clinical isolates. In addition, M. avium complex strains 8624-86, 8626-86 and 8627-86 were transformed with plasmid pMV261 (Stover et al., 1991), identical to pMV262 except for a base pair addition in the multiple cloning site of the latter. M. avium strains 18, MAC100, MAC101 and MAC109 were resistant to 50 μg kanamycin ml⁻¹ and their transformability was not evaluated. Kanamycin-resistant M. avium complex transformants and their parent kanamycin-sensitive strains were all sensitive to 16 μg amikacin ml⁻¹, an aminoglycoside related to kanamycin, which is used as a first-line antimycobacterial agent.

The identities of all M. avium complex transformants were confirmed by a positive test with the AccuProbe M. avium complex probe, and M. paratuberculosis transformants by mycobactin dependence. Many of the strains tested were transformable, at varying efficiencies (Table 4). M. avium complex strains mc576 and 8627-86 gave approximately 10- to 50-fold more transformants than other M. avium complex or M. paratuberculosis strains, but still 10-fold fewer than M. smegmatis. Among M. paratuberculosis strains, the clinical isolate K-10 gave the greatest number of transformants, approximately a fivefold higher transformation efficiency than the prototype strain. Transformation of M. paratuberculosis K-10 with the cloning vector pMV262 and the recombinant pYUB180 gave approximately the same number of transformants, indicating that transformation efficiencies were determined by properties of the host strain rather than the plasmid DNA.

Indigenous plasmids have been reported in M. avium complex strains (Crawford & Falkinham, 1990) which may decrease the transformation efficiencies by a mechanism of incompatibility or competition for replication or partition. Attempts to find plasmids in M. avium complex strains were carried out by a DNA minipreparation procedure as previously described (Jacobs et al., 1991). Approximately 3 x 10⁶ cells (10 ml cultures) were harvested at mid-exponential phase (OD₆₀₀ approximately 0.5). Gel electrophoresis revealed a 2.3 kb plasmid in the transformable strain mc571 and a 14 kb plasmid in the kanamycin-resistant strain MAC109 (data not shown). None of the other M. avium complex strains appeared to have plasmids, although the presence of large low-copy-number plasmids cannot be ruled out.

Characterization of M. avium complex and M. paratuberculosis transformants

To determine the stability and integrity of the shuttle plasmid, DNA was isolated from a representative M. paratuberculosis K-10 transformant expressing the firefly luciferase gene. To facilitate restriction endonuclease
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Fig. 4. Expression of firefly luciferase in M. smegmatis mc²155 (white bars), M. avium mc76 (hatched bars), and M. paratuberculosis K-10 (stippled bars) transformants carrying plasmid pYUB180. Kanamycin-resistant transformants were grown to an OD₆₅₀ of approximately 1.0 (3 × 10⁸ c.f.u. ml⁻¹), tenfold serial dilutions were prepared in duplicate, and luciferase activities were measured. Error bars indicate SD.

DISCUSSION

The lack of systems for the genetic analysis of M. avium complex and M. paratuberculosis has delayed the accurate identification of genes involved in pathogenesis. Progress has been made by cloning M. avium (Rouse et al., 1991) and M. paratuberculosis (Murray et al., 1989) genes into E. coli or M. smegmatis (Belisle et al., 1991), and by the use of subtration hybridization techniques (Plum & Clark-Curtiss, 1994). However, the definitive proof of the involvement of a virulence gene requires the fulfilment of Koch's postulates at the molecular level (Falkow, 1988). Cardinal to this tenet is the construction of isogenic mutant strains and their comparison with the wild-type strain by in vitro virulence assays (Rastogi et al., 1987), and infectivity studies in animal models (Bermudez et al., 1992; Gangadharam et al., 1989; Hamilton et al., 1991). Application of in vivo expression technology (Mahan et al., 1993) to these pathogenic mycobacterial species also requires the construction of deletion mutants and the expression of reporter genes. Therefore, it was of interest to investigate whether the molecular tools developed for M. smegmatis, M. tuberculosis and M. bovis BCG (Jacobs et al., 1991) could also be used for the genetic manipulation of M. avium complex and M. paratuberculosis.

Phage susceptibility studies (Table 2) showed that mycobacteriophage TM4 plaqued at the same efficiency on each mycobacterial host. This suggests either that there are no restriction–modification barriers between M. smegmatis, and M. avium or M. paratuberculosis, or that TM4 may possess a mechanism to overcome restriction–modification barriers. Infection of the fast-growing M. smegmatis and the slow-growing M. avium with the luciferase reporter phage phAE39 gave similar kinetics of light output, suggesting that the rates of transcription and translation are similar in both mycobacterial species and do not account for the different growth rates. This is in agreement with previous findings reported with M. tuberculosis and M. bovis BCG (Jacobs et al., 1993). In contrast, infection of M. paratuberculosis with phAE39 resulted in a slower rate of luciferase production. Differences in the rates of phage absorption and replication, rates of transcription and translation, or levels of ATP needed for the luciferase reaction may explain this effect.

The limited survey of M. avium complex strains for susceptibility to infection with phages phAE39 and phAE40 revealed some differences. Two strains were not susceptible to the mycobacteriophages, while other strains produced varying levels of luciferase. No significant differences were observed between phAE39 and phAE40 (isolated as a host-range mutant capable of infecting M. bovis BCG; Jacobs et al., 1993), with both phages...
generating similar levels of luciferase in the same mycobacterial host.

Transfection with phage DNA (Table 3) was considerably less efficient than transformation with plasmid DNA (Table 4). The large molecular mass of phage DNA (50 kb) may not allow for efficient electroporation across mycobacterial cell walls. Transfection and transformation efficiencies observed with M. avium complex and M. paratuberculosis hosts, however, were between 10- and 1000-fold lower than with M. smegmatis mc²155. M. avium complex and M. paratuberculosis cell walls are probably more resilient to the passage of DNA by electroporation than M. smegmatis, in which case higher efficiencies could potentially be obtained in these strains by a combination of protoplasting methods (Jacobs et al., 1987) and electroporation. Nevertheless, most of the M. avium complex and M. paratuberculosis strains tested were transformed, indicating that these mycobacterial species share the machinery necessary for replication of pAL5000-type shuttle plasmids. These plasmids can also replicate in M. fortuitum (Labidi et al., 1984), M. smegmatis, M. bovis BCG, M. tuberculosis (Jacobs et al., 1991), M. vaccae and Mycobacterium w (Garbe et al., 1994). We have not tested whether the newly developed hygromycin-resistant shuttle plasmids (Garbe et al., 1994) would allow for selection and greater transformation efficiencies in M. avium complex and M. paratuberculosis.

The shuttle plasmid pYUB180 maintained its integrity after sequential transformation into M. paratuberculosis and E. coli, with no apparent rearrangement after one passage in M. paratuberculosis (Fig. 3). Expression of the firefly luciferase in pYUB180 M. avium and M. paratuberculosis transformants was determined and compared to the luciferase expression from pYUB180 M. smegmatis transformants (Fig. 4). The results indicated that the luciferase gene was expressed from the heat-shock promoter to approximately the same level, suggesting that the transcriptional machineries from these microorganisms share substantial similarities.

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