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

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***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 phasmids 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 late 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 IS*900* (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 phasmids is demonstrated. We also report the replication of shuttle plasmids derived from the *M. fortuitum* plasmid pAL5000 (Labidi *et al.*, 1984; Snapper *et al.*, 1988; Stover *et al.*, 1991), and the isolation of plasmid transformants expressing the firefly luciferase gene. These tools should make possible the genetic manipulation of these micro-organisms 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 (Iddexx 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 15 g l^{-1}. High-titre lysates were prepared and purified by caesium chloride equilibrium density centrifugation, as previously described (Jacobs *et al.*, 1991).

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 $3·0 \times 10^7$ 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 phasmids 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 (OD_{600} 0·2; approximately $6·0 \times 10^7$ 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 OD_{600} of approximately 0·1. From these cultures 1·0 ml (approx. $3·0 \times 19^7$ c.f.u.) was infected with $1·0 \times 10^9$ 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 $1·0 \times 10^{10}$ 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 Ω, 25 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 $5·0 \times 10^7$ 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. *M. avium* complex and *M. paratuberculosis* plaques and colonies developed in 4 to 6 weeks.

Standard DNA procedures. Procedures for plasmid isolation from *E. coli* and mycobacterial hosts, agarose gel electrophoresis, restriction endonuclease analysis and transformation of *E. coli* with plasmid DNA were as described elsewhere (Barletta *et al.*, 1992; Jacobs *et al.*, 1991; Kado & Liu, 1981; Sambrook *et al.*, 1989). Procedures to detect *M. avium* complex DNA by hybridization with AccuProbe *M. avium* complex probe (Gen-Probe) were carried out as described by the manufacturer.

Antibiotic susceptibility assays. To determine the minimum concentration of kanamycin necessary for selection of *M. avium* complex and *M. paratuberculosis* transformants, approximately $5·0 \times 10^9$ 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 (Inderlied *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–scrofulaceum* complex (Crawford & Falkinham, 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

Strain, phage or plasmid	Source and/or reference and relevant characteristics
Strains	
E. coli	
DH5α	Gibco BRL; *recA lacZΔM15*
M. smegmatis	
mc²155	W. R. Jacobs Jr (Albert Einstein College of Medicine, New York) Snapper *et al.* (1990) high-efficiency transformation mutant
M. paratuberculosis	
ATCC 19698	Merkal (1979)
K-10	This study
S-23	This study
M. avium complex	
Strain 18	Sneath & Skerman (1966); Thorel *et al.* (1990); *M. avium* serovar 2, ex *M. paratuberculosis* strain 18
mc²71	W. R. Jacobs Jr; originally from P. J. Brennan's collection (Colorado State University); *M. avium* Bridge, serovar 2
mc²74	W. R. Jacobs Jr; *M. avium* 158, serovar 2
mc²76	W. R. Jacobs Jr; *M. avium* TMC 1419, serovar 2
mc²77	W. R. Jacobs Jr; *M. avium* TMC 1461, serovar 2
MAC100	Inderlied *et al.* (1993); isolated from AIDS patient, *M. avium*, serovar 8
MAC101	Inderlied *et al.* (1993); isolated from AIDS patient, *M. avium*, serovar 1
MAC109	Inderlied *et al.* (1993); isolated from AIDS patient, *M. avium*, serovar 4
8624-86	R. C. Good (Centers for Disease Control and Prevention, Atlanta); *M. intracellulare*, serovar 14
8626-86	R. C. Good; *M. intracellulare*, serovar 16
8627-86	R. C. Good; *M. intracellulare*, serovar 16
Mycobacteriophages	
TM4	Timme & Brennan (1984)
D29	Froman *et al.* (1954)
ph60	W. R. Jacobs Jr; soil isolate
ph72	W. R. Jacobs Jr; soil isolate
phAE39	Jacobs *et al.* (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 *E. coli*, plaques at low efficiency on BCG
phAE40	Jacobs *et al.* (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 *E. coli*, plaques at high efficiency on BCG
Bxb1	Barletta *et al.* (1992)
L1	Snapper *et al.* (1988)
Plasmids	
pMV261	MedImmune (Gaithersburg, MD, USA); Stover *et al.* (1991); 4·5 kb, kanamycin-resistance, replicates in *E. coli* and mycobacteria
pMV262	MedImmune; Connell *et al.* (1993); 4·5 kb, kanamycin-resistance, replicates in *E. coli* and mycobacteria
pYUB180	Jacobs *et al.* (1993); 6·5 kb, kanamycin-resistance, carries the firefly luciferase gene downstream from pMV261 *hsp60* promoter, replicates in *E. coli* and mycobacteria

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.

Phage	Plaquing efficiency (p.f.u. ml^{-1}) of:			
	M. smegmatis	*M. paratuberculosis*		
	mc²155	ATCC 19698	K-10	S-23
Phages propagated on *M. smegmatis* mc²155				
TM4	$5{\cdot}2\pm0{\cdot}2\times10^{10}$	$9\pm2\times10^{10}$	$7\pm2\times10^{10}$	$6{\cdot}0\pm0{\cdot}8\times10^{10}$
D29	$1{\cdot}5\pm0{\cdot}1\times10^{9}$	None	None	None
ph60	$2{\cdot}2\pm0{\cdot}1\times10^{9}$	20 ± 10	$4{\cdot}8\pm0{\cdot}7\times10^{8}$	$3{\cdot}1\pm0{\cdot}6\times10^{5}$
ph72	$5{\cdot}8\pm0{\cdot}2\times10^{9}$	None	None	None
phAE39	$8{\cdot}7\pm0{\cdot}7\times10^{10}$	$4{\cdot}4\pm0{\cdot}7\times10^{10}$	$4{\cdot}3\pm0{\cdot}5\times10^{9}$	$4{\cdot}4\pm0{\cdot}5\times10^{9}$
Bxb1	$4{\cdot}8\pm0{\cdot}2\times10^{9}$	None	None	None
L1	$1{\cdot}8\pm0{\cdot}1\times10^{9}$	None	None	None
Phages propagated on *M. paratuberculosis* (phage/propagation host)				
TM4/K-10	$2{\cdot}5\pm0{\cdot}1\times10^{7}$	$1{\cdot}4\pm0{\cdot}3\times10^{7}$	$1{\cdot}4\pm0{\cdot}4\times10^{7}$	$2{\cdot}0\pm0{\cdot}3\times10^{7}$
TM4/ATCC 19698	$9{\cdot}9\pm0{\cdot}2\times10^{7}$	$2{\cdot}7\pm0{\cdot}5\times10^{7}$	$1{\cdot}7\pm0{\cdot}4\times10^{7}$	$2{\cdot}1\pm0{\cdot}4\times10^{7}$
TM4/S-23	$2{\cdot}6\pm0{\cdot}1\times10^{7}$	$2{\cdot}4\pm0{\cdot}3\times10^{7}$	$2{\cdot}3\pm0{\cdot}3\times10^{7}$	$1{\cdot}7\pm0{\cdot}3\times10^{7}$

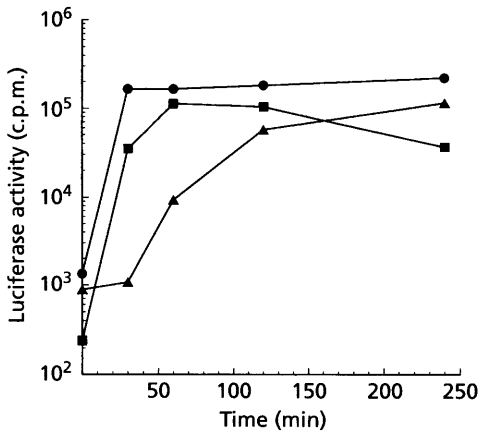

Fig. 1. Kinetics of light output resulting from infection of *M. smegmatis* mc²155 (●), *M. avium* mc²76 (■) and *M. paratuberculosis* K-10 (▲) with the luciferase reporter phage phAE39. Data indicate the luciferase activities from a representative experiment.

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.*,

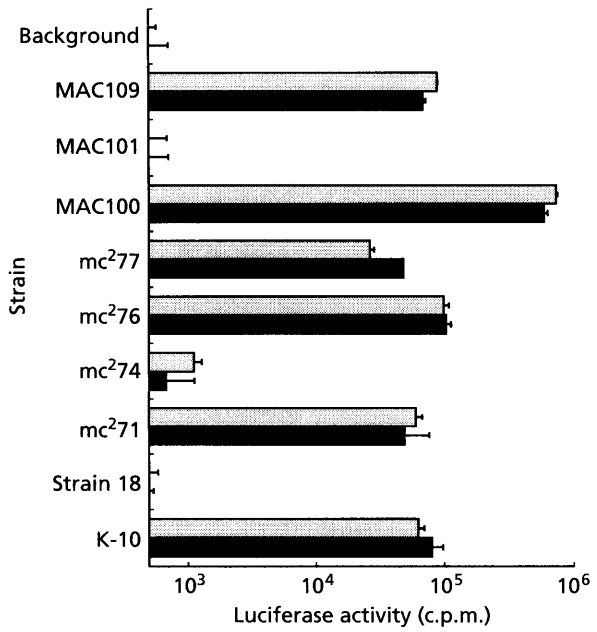

Fig. 2. Survey of *M. avium* complex strains for luciferase production upon infection with the luciferase reporter phages phAE39 (stippled bars) and phAE40 (solid bars). Luciferase activities at 120 min after infection were determined. Infections of *M. paratuberculosis* K-10 and *M. avium* mc²76 were used as positive controls. The background luciferase activity represents the mean activity from all assays determined at the start of phage infections. Error bars indicate SD of duplicate sample measurements from a single experiment.

1989; Taylor *et al.*, 1987). This technology requires a sensitive reporter gene assay. Since the firefly luciferase assays are highly sensitive and the luciferase gene has been expressed in *M. smegmatis*, BCG and *M. tuberculosis* (Cooksey *et al.*, 1993; Jacobs *et al.*, 1993), we evaluated the expression of the firefly luciferase gene in *M. avium* complex and *M. paratuberculosis* by the use of the luciferase reporter mycobacteriophages phAE39 and phAE40 (Jacobs *et al.*, 1993). Infection of *M. avium* mc²76 and *M. paratuberculosis* K-10 with the TM4 derivatives phAE39 and phAE40 resulted in light production. The kinetics of light output generated upon infection of *M. paratuberculosis* with phAE39 was somewhat slower than with *M. avium* and *M. smegmatis* as hosts (Fig. 1), although all strains reached approximately the same maximum level of luciferase activity at 120 min after phage infection.

Reporter phage assays were also performed with *M. avium* complex strains including isolates from AIDS patients (Fig. 2). The serovar 2 strains mc²71, mc²76 and mc²77, and the AIDS patient isolates MAC100 (serovar 8) and MAC109 (serovar 4) resulted in moderate to high levels of luciferase activity with both phAE39 and phAE40. The serovar 2 strain mc²74 produced slightly above background bioluminescence upon infection with phAE39 and no light output upon infection with phAE40. The serovar 2 strain 18 and the serovar 1 strain MAC101 were not infected by phAE39 or phAE40 and did not produce a light output. The *M. avium* complex strains 8624-86, 8626-86 and 8627-86 were not tested in this survey.

Table 3. Transfection of *M. paratuberculosis*

Transfection efficiencies were determined by counting the number of isolated plaques resulting from transfection with 5·0 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

Strain	Phage DNA	OD_{600} of culture at harvest	Transfection efficiency (p.f.u.)
M. smegmatis mc²155	D29	0·7	396 ± 20
	TM4	0·7	132 ± 12
M. paratuberculosis K-10	D29	0·5	None
	TM4	0·3	16 ± 5
	TM4	0·5	4 ± 2
	TM4	1·0	2 ± 1
	TM4	1·0*	None

*Culture was harvested in stationary phase, after OD_{600} had remained at 1·0 for approximately 1 week.

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 Tn*903* (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.

Strain	Plasmid DNA	OD_{600} of culture at harvest	Transformation efficiency [c.f.u. (μg DNA)$^{-1}$]
M. smegmatis			
mc^{2}155	pYUB180	0·50	$2{\cdot}8 \pm 0{\cdot}2 \times 10^5$*
mc^{2}155	pYUB180	0·50	$3{\cdot}1 \pm 0{\cdot}8 \times 10^5$
M. paratuberculosis			
ATCC 19698	pYUB180	0·20	$1{\cdot}4 \pm 0{\cdot}1 \times 10^2$
K-10	pMV262	0·23	$7{\cdot}1 \pm 0{\cdot}3 \times 10^2$
K-10	pYUB180	0·23	$6{\cdot}5 \pm 0{\cdot}3 \times 10^2$
S-23	pYUB180	0·20	$6{\cdot}3 \pm 0{\cdot}3 \times 10^2$
***M. avium* complex**			
mc^{2}71	pYUB180	0·20	$1{\cdot}6 \pm 0{\cdot}4 \times 10^3$
mc^{2}74	pYUB180	0·20	30 ± 5
mc^{2}76	pYUB180	0·20	$1{\cdot}0 \pm 0{\cdot}1 \times 10^4$
mc^{2}77	pYUB180	0·20	None
8624-86	pMV261	0·24	$7 \pm 2 \times 10^2$
8626-86	pMV261	0·23	$3{\cdot}4 \pm 0{\cdot}3 \times 10^3$
8627-86	pMV261	0·21	$2{\cdot}10 \pm 0{\cdot}02 \times 10^4$

* Transformants 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 mc^{2}77, 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 mc^{2}76 and 8627-86 gave approximately

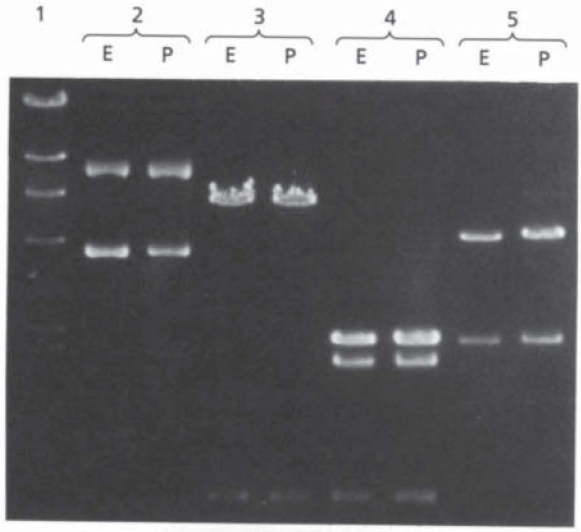

Fig. 3. Agarose gel (0·8%) analysis of plasmids isolated from the original *E. coli* pYUB180 transformant (lanes E), and those recovered from a *M. paratuberculosis* K-10 pYUB180 transformant (lanes P). 1, Bacteriophage λ DNA digested with *Hin*dIII; 2, uncut pYUB180; 3, *Eco*RI-cut pYUB180; 4, *Eco*RI- and *Sma*I-cut pYUB180; 5, *Sma*I-cut pYUB180.

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 $3{\cdot}0 \times 10^9$ cells (10 ml cultures) were harvested at mid-exponential phase (OD_{600} approximately 0·5). Gel electrophoresis revealed a 2·3 kb plasmid in the transformable strain mc^{2}71 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

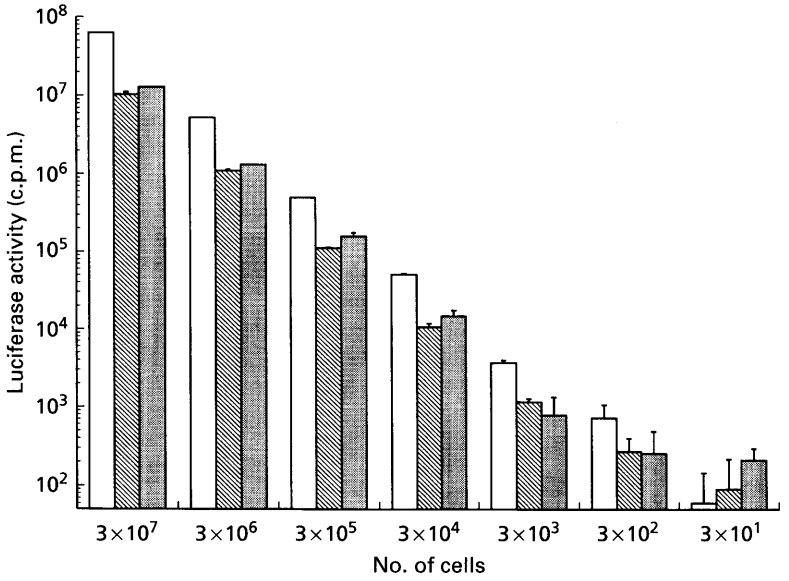

Fig. 4. Expression of firefly luciferase in *M. smegmatis* mc^{2}155 (white bars), *M. avium* mc^{2}76 (hatched bars), and *M. paratuberculosis* K-10 (stippled bars) transformants carrying plasmid pYUB180. Kanamycin-resistant transformants were grown to an OD_{600} of approximately 1·0 ($3{\cdot}0\times10^8$ c.f.u. ml^{-1}), tenfold serial dilutions were prepared in duplicate, and luciferase activities were measured. Error bars indicate SD.

analysis, the shuttle plasmid isolated from *M. paratuberculosis* was transformed back into *E. coli*. Plasmid DNA was purified from the original *E. coli* clone and from *E. coli* transformed with plasmid DNA isolated from *M. paratuberculosis*, and digested with *Eco*RI or *Sma*I, either separately or in combination. Restriction fragments were separated by agarose gel electrophoresis. Digestion patterns from each plasmid were identical (Fig. 3). Therefore, within the conditions described, no apparent loss of pYUB180 sequences was observed in *M. paratuberculosis* K-10 transformants.

Expression of the firefly luciferase was compared in *M. smegmatis*, *M. avium* and *M. paratuberculosis* pYUB180 transformants grown in Middlebrook 7H9 medium. Quantitative analysis was performed to determine the minimum number of cells detectable by the luciferase assay (Fig. 4). The luciferase gene was expressed at similar levels in the three mycobacterial species. Serial dilutions indicated that the luciferase assay detected 300 to 3000 *M. avium* or *M. paratuberculosis* cells expressing firefly luciferase, establishing that the firefly luciferase is a sensitive reporter gene to measure the viability of *M. avium* and *M. paratuberculosis* cells.

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 subtraction 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 electrotransfer 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* mc^2155. *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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REFERENCES

Adams, M. H. (1959). *Bacteriophages.* New York: Interscience Publishers.

Barletta, R. G., Kim, D. D., Snapper, S. B., Bloom, B. R. & Jacobs, W. R., Jr (1992). Identification of expression signals of the mycobacteriophages Bxb1, L1, and TM4 using *Escherichia–Mycobacterium* shuttle plasmids pYUB75 and pYUB76 designed to create translational fusions to the *lacZ* gene. *J Gen Microbiol* **138**, 23–30.

Belisle, J. T., Pascopella, L., Inamine, J., Brennan, P. J. & Jacobs, W. R., Jr (1991). Isolation and expression of a gene cluster responsible for biosynthesis of the glycopeptidolipid antigens of *Mycobacterium avium*. *J Bacteriol* **173**, 6991–6997.

Bermudez, L. E., Petrofsky, M., Kolonoski, P. & Young, L. S. (1992). An animal model of *Mycobacterium avium* complex disseminated infection after colonization of the intestinal tract. *J Infect Dis* **165**, 75–79.

Chiodini, R. J., Van Kruiningen, H. J. & Merkal, R. S. (1984). Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet* **74**, 218–262.

Connell, N. D., Medina-Acosta, E., McMaster, W. R., Bloom, B. R. & Russell, D. G. (1993). Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guérin expressing the *Leishmania* surface proteinase gp63. *Proc Natl Acad Sci USA* **90**, 11473–11477.

Cooksey, R. C., Crawford, J. T., Jacobs, W. R. & Shinnick, T. M. (1993). A rapid method for screening antimicrobial agents for activities against a strain of *Mycobacterium tuberculosis* expressing firefly luciferase. *Antimicrob Agents Chemother* **37**, 1348–1352.

Crawford, J. T. & Falkinham, I. J. O. (1990). Plasmids of the *Mycobacterium avium* complex. In *Molecular Biology of the Mycobacteria*, pp. 97–119. Edited by J. J. McFadden. London: Academic Press.

Dorman, C. J., Bhriain, N. N. & Higgins, C. F. (1990). DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. *Nature* **344**, 789–792.

Falkow, S. (1988). Molecular Koch's postulates applied to microbial pathogenicity. *Rev Infect Dis* **10**, S274–S276.

Froman, S., Will, W. & Bogen, E. (1954). Bacteriophages active against virulent *Mycobacterium tuberculosis*. Isolation and activity. *Am J Public Health* **44**, 1326–1334.

Galán, J. E. & Curtiss, R., III (1990). Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. *Infect Immun* **58**, 1879–1885.

Gangadharam, P. R. J., Perumal, V. K., Farhi, D. C. & LaBrecque, J. (1989). The beige mouse model for *Mycobacterium avium* complex disease: optimal conditions for the host and the parasite. *Tubercle* **70**, 257–271.

Garbe, T. R., Barathi, J., Barnini, S., Zhang, Y., Abou-Zeid, C., Tang, D., Mukherjee, R. & Young, D. B. (1994). Transformation of mycobacterial species using hygromycin resistance as selectable marker. *Microbiology* **140**, 133–138.

Green, E. P., Tizard, M. L. V., Moss, M. T., Thompson, J., Winterbourne, D. J., McFadden, J. J. & Hermon-Taylor, J. (1989). Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res* **17**, 9063–9073.

Hamilton, H. L., Cooley, A. J., Adams, J. L. & Czuprynski, C. J. (1991). *Mycobacterium paratuberculosis* monoassociated nude mice as a paratuberculosis model. *Vet Pathol* **28**, 146–155.

Hawkins, C. C., Gold, J. W., Whimbey, E., Kiehn, T. E., Brannon, P., Cammarata, R., Brown, A. E. & Armstrong, D. (1986). *Mycobacterium avium* complex infections in patients with acquired immunodeficiency syndrome. *Ann Intern Med* **105**, 184–188.

Horsburgh, C. R., Jr, Mason, U. G., III, Farhi, D. C. & Iseman, M. D. (1985). Disseminated infection with *Mycobacterium avium–intracellulare*: a report of 13 cases and a review of the literature. *Medicine* **64**, 36–48.

Hurley, S. S., Splitter, G. A. & Welch, R. A. (1988). Deoxyribonucleic acid relatedness of *Mycobacterium paratuberculosis*

to other members of the family *Mycobacteriaceae*. *Int J Syst Bacteriol* **38**, 143–146.

Inderlied, C. B., Young, L. S. & Yamada, J. K. (1987). Determination of in vitro susceptibility of *Mycobacterium avium* complex isolates to antimicrobial agents by various methods. *Antimicrob Agents Chemother* **31**, 1697–1702.

Inderlied, C. B., Kemper, C. A. & Bermudez, L. E. M. (1993). The *Mycobacterium avium* complex. *Clin Microbiol Rev* **6**, 266–310.

Jacobs, W. R., Jr, Tuckman, M. & Bloom, B. R. (1987). Introduction of foreign DNA into mycobacteria using a shuttle plasmid. *Nature* **327**, 532–535.

Jacobs, W. R., Jr, Ganjam, K. V., Cirillo, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., Jones, W., Barletta, R. G. & Bloom, B. R. (1991). Genetic systems for the mycobacteria. *Methods Enzymol* **204**, 537–555.

Jacobs, W. R., Jr, Barletta, R. G., Udani, R., Chan, J., Kalkut, G., Sosne, G., Kieser, T., Sarkis, G. J., Hatfull, G. F. & Bloom, B. R. (1993). Rapid assessment of susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* **260**, 819–822.

Kado, C. & Liu, S. (1981). Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* **145**, 1365–1373.

Labidi, A., Dauguet, C., Goh, K. S. & David, H. (1984). Plasmid profiles of *Mycobacterium fortuitum* complex isolates. *Curr Microbiol* **11**, 235–240.

Lugosi, L., Jacobs, W. R., Jr & Bloom, B. R. (1989). Genetic transformation of BCG. *Tubercle* **70**, 159–170.

Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686–688.

Martín, C., Ranes, M. & Gicquel, B. (1990). Plasmids, antibiotic resistance, and mobile genetic elements in mycobacteria. In *Molecular Biology of the Mycobacteria*, pp. 121–138. Edited by J. J. McFadden. London: Academic Press.

McFadden, J. J., Butcher, P. D., Chiodini, R. & Hermon-Taylor, J. (1987). Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol* **25**, 796–801.

Miller, V. L., Taylor, R. K. & Mekalanos, J. J. (1987). Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding domain. *Cell* **48**, 271–279.

Merkal, R. S. (1979). Proposal of ATCC 19698 as the neotype strain of *Mycobacterium paratuberculosis* Bergey *et al.* 1923. *Int J Syst Bacteriol* **29**, 263–264.

Murphy, G. L., Connell, T. D., Barritt, D. S., Koomey, M. & Cannon, J. G. (1989). Phase variation of gonococcal protein II, regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. *Cell* **56**, 539–547.

Murray, A., Moriarty, K. M. & Scott, D. B. (1989). A cloned DNA probe for the detection of *Mycobacterium paratuberculosis*. *NZ Vet J* **37**, 47–50.

Oka, A., Sugisaki, H. & Tanakami, M. (1981). Nucleotide sequence of the kanamycin-resistance transposon Tn*903*. *J Mol Biol* **147**, 217–226.

Plum, G. & Clark-Curtiss, J. E. (1994). Induction *Mycobacterium avium* gene expression following phagocytosis by human macrophages. *Infect Immun* **62**, 476–483.

Rastogi, N., Potar, M.-C. & David, H. L. (1987). Intracellular growth of pathogenic mycobacteria in the continuous murine macrophage cell line J774: ultrastructure and drug-susceptibility studies. *Curr Microbiol* **16**, 79–92.

Rouse, D. A., Morris, S. L., Karpas, A. B., MacKall, J. C., Probst, P. G. & Chaparas, S. D. (1991). Immunological characterization of recombinants antigens isolated from a *Mycobacterium avium* lambda gt11 expression library by using monoclonal antibody probes. *Infect Immun* **59**, 2595–2600.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sneath, P. H. A. & Skerman, V. B. D. (1966). A list of type and reference strains of bacteria. *Int J Syst Bacteriol* **29**, 263–264.

Snapper, S. B., Lugosi, L., Jekkel, A., Melton, R. E., Kieser, T., Bloom, B. R. & Jacobs, W. R., Jr (1988). Lysogeny and transformation in mycobacteria: stable expression of foreign genes. *Proc Natl Acad Sci USA* **85**, 6987–6991.

Snapper, S. B., Melton, R. E., Kieser, T., Mustafa, S. & Jacobs, W. R., Jr (1990). Isolation and characterization of high efficiency plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* **4**, 1911–1919.

Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H., Hatfull, G. F., Snapper, S. B., Barletta, R. G., Jacobs, W. R., Jr & Bloom, B. R. (1991). New use of BCG for recombinant vaccines. *Nature* **351**, 456–460.

Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA* **84**, 2833–2837.

Thorel, M. F., Krichevsky, M. & Lévy-Frébault, V. V. (1990). Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int J Syst Bacteriol* **40**, 254–260.

Timme, T. L. & Brennan, P. J. (1984). Induction of bacteriophage from members of the *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum* serocomplex. *J Gen Microbiol* **130**, 2059–2066.

Young, L. S. (1988). *Mycobacterium avium* complex infection. *J Infect Dis* **157**, 863–866.

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