Enhanced gene replacement in mycobacteria

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Allelic replacement will be a vital tool for understanding gene function in mycobacteria. Disruption of the chromosomal *hisD* gene of *Mycobacterium smegmatis* by standard gene replacement methods was surprisingly difficult, with most products being caused by illegitimate recombination (IR) events. A recombination assay was therefore developed and used to optimize conditions for homologous recombination (HR) in *M. smegmatis*. Treatment of competent cells with UV, hydrogen peroxide or mitomycin C did not improve the frequency of HR; however, treatment of the DNA with alkali or UV enhanced recombination frequency, while boiling did not. Applying these observations to allele replacement, UV and alkali treatment of transforming DNA increased HR events with *pyrF* and *hisD*, while the level of IR was unchanged. The introduction of ss phagemid DNA improved the level of HR and abolished IR. In *Mycobacterium intracellulare* the use of alkali-denatured DNA increased the numbers of recombinants obtained with an inactivated 19Ag gene, while in *Mycobacterium tuberculosis*, inactivation of a putative haemolysin gene, *tlyA*, was achieved using both UV-irradiated DNA and ss phagemid DNA. Significantly, IR, which has been reported to be a problem in this species, was not observed. Thus, four genes in three species were successfully knocked-out using non-replicating DNA pretreated with alkali, UV or in an ss form. The use of these methods to enhance HR will greatly facilitate experiments to inactivate other genes in these important species.

Keywords: alkali-treated DNA, UV-irradiated DNA, haemolysin, homologous recombination, illegitimate recombination

INTRODUCTION

Mycobacterial disease is a major public health problem throughout the world (Raviglione *et al.*, 1995). Development of new rational methods to control these diseases will depend on a greater understanding of the biology of the pathogens. An essential tool for understanding the biology of these organisms is the ability to inactivate genes at will, particularly as a large proportion of the genes have no known function (Cole *et al.*, 1998).

Targeted gene replacement is routine in many bacteria, but has proved to be difficult in mycobacteria (McFadden, 1996). Work by Husson *et al.* (1990) showed that *pyrF* mutants could be readily obtained by trans-

forming *Mycobacterium smegmatis* with a non-replicating plasmid carrying *pyrF* disrupted by a kanamycin resistance gene (*kan*). Kalpana *et al.* (1991) also successfully inactivated *M. smegmatis* genes using a gene library in an *Escherichia coli* plasmid vector, which was mutagenized with Tn5 and then transformed into the mycobacterium. However, when they transformed *Mycobacterium tuberculosis* with linear DNA, all the antibiotic-resistant transformants had arisen through illegitimate recombination (IR), with the fragment integrating at different sites in the chromosome in an apparently random manner. IR is rare in prokaryotes, but common in eukaryotic cells and this suggested that slow-growing mycobacteria such as *M. tuberculosis* might be unusual in having a high level of IR, perhaps accompanied by a depressed level of homologous recombination (HR). This idea was strengthened by the discovery of inteins in the *recA* genes of *M. tuberculosis* and *Mycobacterium leprae* (Davis *et al.*, 1994). This

Abbreviations: HR, homologous recombination; IR, illegitimate recombination; SCO, single cross-over; DCO, double cross-over.

again suggested that RecA activity and hence the level of HR might be suppressed, with the high level of IR being explained by the persistence of chromosomal breaks (McFadden, 1996), although this now seems unlikely (Papavinasundaram *et al.*, 1998). The first evidence that gene replacement was possible in slow-growing mycobacteria was in *Mycobacterium intracellulare* using a two-step selection procedure (Marklund *et al.*, 1995) and in *Mycobacterium bovis* BCG using either a replicating vector (Norman *et al.*, 1995) or a linearized-suicide vector (Aldovini *et al.*, 1993).

This paper describes experiments to inactivate *M. smegmatis* genes and the use of a recombination assay to identify conditions in which HR is enhanced. These results were then applied to gene replacement experiments in *M. smegmatis*, *M. intracellulare* and *M. tuberculosis*.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in the work described here are listed in Table 1. *Escherichia coli* strains were cultured as described by Sambrook *et al.* (1989). Mycobacteria were grown in Lemco broth (Lab Lemco powder, 5 g l⁻¹; Bacto peptone, 5 g l⁻¹; NaCl, 5 g l⁻¹; 0.05% Tween 80; used for *M. smegmatis* only) or on Middlebrook 7H10 agar or 7H9 broth (Difco) containing oleic acid dextrose complex (OADC) without added catalase. Gentamicin was added at 5 µg ml⁻¹, hygromycin B at 100 µg ml⁻¹ and kanamycin at 15 µg ml⁻¹ where appropriate.

Electroporation of mycobacteria. *M. smegmatis* was electroporated as described by Snapper *et al.* (1990) and Parish & Stoker (1995). *M. intracellulare* was prepared for electroporation as described previously (Marklund *et al.*, 1995) except that all washes were carried out at room temperature. *M. tuberculosis* was electroporated as described previously (Parish & Stoker, 1995) except that all washes were carried out at room temperature (Wards & Collins, 1996).

DNA purification and manipulations. Purification of plasmid DNA from *E. coli* and all DNA manipulations were carried out using standard procedures (Sambrook *et al.*, 1989). ssDNA was prepared using the protocols provided for the pGEM system (Promega) in *E. coli* JM109. Plasmid DNA was prepared from mycobacteria using mechanical lysis of the bacteria with glass beads followed by DNA purification using the Wizard Miniprep System (Promega). Genomic DNA was isolated from *M. smegmatis* and *M. tuberculosis* by the method of Santos *et al.* (1992). Chromosomal DNA was extracted from *M. intracellulare* for PCR analysis using a miniprep method (Lee *et al.*, 1991).

PCR amplification. Primers HISP1 (5' TATAGATCTGGAC-TGATCAGGGACAATG 3'), complementary to nt 280–307, and HISP6 (5' AGACATTACCGCTCGAA 3'), complementary to nt 1647–1631 of the *M. smegmatis* *hisD* sequence (accession no. X65542; Hinshelwood & Stoker, 1992), were used to amplify a 1367 bp fragment containing the coding sequence of the *hisD* gene. Amplification was carried out in 50 µl containing 50 pmol each primer, 0.2 mM each dNTP, 2.5 units Biotaq polymerase (Bioline), 5 µl 10× buffer (Bioline) containing 15 mM Mg²⁺ and 1 ng cosmid pCMH13 DNA (Hinshelwood & Stoker, 1992). PCR was carried out using 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 70 °C for 1 min. The resulting product was cloned into the TA cloning vector pCRII to create pSYCH03. The amplification pattern obtained with PCR primers BIM-9 (5' AACAAAGTCGGGAACGAGCG 3') and BIM-10 (5' TTGATTTCGAACGGTTTG 3'), which flanked the *Afl*III site of 19Ag gene disruption (see Fig. 1d), was used as a screen for the type of recombination event undergone with plasmid p19K5 (Mahenthiralingam *et al.*, 1998).

Preparation of plasmid DNA for electroporation. Plasmid DNA (1–2 µg) was denatured in 20 µl 0.2 M NaOH containing 0.2 mM EDTA for 30 min at 37 °C. Two microlitres of 3 M sodium acetate, pH 4.8, and 80 µl ethanol were added and the DNA precipitated at –70 °C. The DNA pellet was washed twice with 70% ethanol, dried under vacuum and dissolved in 10 µl sterile deionized water. ssDNA was kept on ice (1–2 h) or frozen for storage at –20 °C, prior to electroporation.

Table 1. Strains and plasmids

Strain or plasmid	Relevant features	Source
<i>E. coli</i> DH5α	<i>recA</i>	Life Technologies
<i>E. coli</i> JM109	F ⁺ (used for ssDNA preparation)	Promega
<i>M. smegmatis</i> mc ² 155		Snapper <i>et al.</i> (1990)
<i>M. tuberculosis</i> H37Rv		Laboratory stock
<i>M. intracellulare</i> 1403 (ATCC 35716)		Trudeau Collection
pCRII	PCR cloning vector	Invitrogen
pGEM3Zf(+)	Phagemid vector	Promega
pSYCH03	1.4 kb <i>hisD</i> PCR product in pCRII	This work
pSYCH09	<i>hisD</i> :: <i>hyg</i> suicide vector	This work
pAGAN40	<i>hyg E. coli</i> –mycobacteria shuttle vector	This work
pRAM4	Recombination assay vector	This work
pCIG19	2.4 kb <i>M. tuberculosis</i> DNA containing <i>tlyA</i> in pUC18	Wren <i>et al.</i> (1998)
pHLY3	<i>tlyA</i> :: <i>hyg</i> suicide vector	This work
p19K5	19Ag:: <i>Gm</i> suicide vector	Mahenthiralingam <i>et al.</i> (1998)
pY6002	<i>pyrF</i> :: <i>kan</i> suicide vector	Husson <i>et al.</i> (1990)

For UV treatment of DNA, plasmid DNA was subjected to 25, 100 or 300 mJ UV irradiation cm⁻² in a UV Stratalinker 1800 (Stratagene). Boiling treatment involved incubating plasmid DNA in a boiling water bath for 15 min followed by 10 min cooling on ice. ss phagemid DNA was generated from *E. coli* JM109 using helper phage R408 following protocols provided with the pGEM Single Strand System (Promega). Purification of ss phagemid DNA was performed using the Wizard M13 DNA Purification System (Promega).

Recombination assay. pRAM4 DNA was prepared from a *recA*-deficient strain of *E. coli*, such as DH5 α , and 1 μ g was electroporated into *M. smegmatis*. After a 2 h recovery incubation, the bacteria were plated onto selective plates containing either hygromycin, kanamycin or hygromycin plus kanamycin. For treatment with DNA damaging agents, the cells were treated immediately after transformation with either UV irradiation at 45, 100, 200 and 400 mJ cm⁻², hydrogen peroxide at 44, 88 and 132 mM or mitomycin C at 0.005, 0.05 and 0.5 μ g ml⁻¹. Treatment with hydrogen peroxide and mitomycin C was for the 2 h recovery period, following which the cells were washed prior to plating.

RESULTS

Gene replacement in *M. smegmatis*

Other groups have readily obtained HR in *M. smegmatis* and we repeated experiments using the suicide vector pY6002 containing the *M. smegmatis* *pyrF* gene disrupted by insertion of a *kan* gene (Husson *et al.*, 1990). It was introduced into *M. smegmatis* as either circular DNA or as a linear fragment (an *EcoRI*–*XbaI* fragment); colonies were obtained with all substrates and analysed. For *pyrF*, 12/194 (6%) of the transformants from circular DNA were uracil auxotrophs, compared to 3/72 (4%) with linear DNA. One auxotroph and five prototrophs were analysed by Southern blotting (not shown). The uracil auxotrophs showed the expected pattern for a double cross-over (DCO); of the prototrophs, 4/5 had undergone a single cross-over (SCO) HR event, while 1/5 had arisen through IR. Thus, using *pyrF*, HR was readily achieved, with the majority of events being SCOs.

Having obtained HR with *pyrF*, we carried out similar experiments with the *M. smegmatis* *hisD* gene encoding histidinol dehydrogenase (Hinshelwood & Stoker, 1992), which catalyses the final two steps in histidine biosynthesis (Alifano *et al.*, 1996). pSYCH09, a non-replicating (suicide) vector containing the *hisD* gene disrupted by insertion of a hygromycin resistance gene (*hyg*) (Fig. 1c) was electroporated into *M. smegmatis* and low numbers of hygromycin-resistant (Hyg^R) transformants were obtained (0–25 μ g DNA). One hundred and forty six transformants were obtained from several electroporations; however, none was auxotrophic and all of those examined by Southern blotting were found to be IR events (not shown; these transformants are currently being characterized). We concluded that the level of HR with the *hisD* construct was low, such that it was below the background level of IR, because of either the gene itself or some technical factor such as the length of flanking DNA. Therefore, we carried out

experiments to improve the system, both for ‘difficult’ genes in *M. smegmatis* and for *M. tuberculosis*, where gene replacement has proved more troublesome.

Recombination assays in *M. smegmatis*

To identify experimental conditions for application in gene replacement experiments that favoured HR, a plasmid-based recombination assay was developed which would generate quantitative results. The recombination assay vector, pRAM4, was constructed based upon restoration of a functional *hyg* gene by an HR event between two overlapping fragments of the *hyg* gene flanking a functional *kan* gene in a shuttle plasmid (Fig. 1a, b). Intraplasmid HR between the duplicated DNA would restore a complete and functional *hyg* gene, deleting the *kan* cassette in the process. Neither of the fragments of *hyg* used could confer resistance to hygromycin on their own.

Kan^R pRAM4 transformants were picked and grown in Lemco broth containing kanamycin and plated onto Lemco plates containing kanamycin, hygromycin or kanamycin plus hygromycin. Transformants were obtained on all the selective media used, indicating that the native (Kan^R) pRAM4 vector was able to rearrange by HR to produce Hyg^R colonies. Plasmid DNA was isolated from transformants and digested with restriction enzymes. The plasmids from Hyg^R and Kan^R colonies had the expected structures, while plasmid DNA isolated from the double antibiotic selection indicated that these were colonies in which both native and recombined forms of pRAM4 were present, which we would expect to segregate in the absence of a double antibiotic selection (not shown). The mean frequency of recombination using untreated plasmid was 4.6×10^{-3} ($n = 8$; $\sigma = 4.7 \times 10^{-3}$).

Attempts to improve the level of HR by modification to the standard transformation protocol did not produce any significant benefits. Modifications assessed included the use of freshly prepared rather than frozen competent *M. smegmatis* and cells prepared at different growth stages. Treatment of transformed *M. smegmatis* with DNA damaging agents was also evaluated using the recombination assays. UV irradiation of transformed *M. smegmatis* at energies of 45–400 mJ cm⁻² produced no significant effect upon recombination frequencies or transformant numbers (not shown). Hydrogen peroxide treatment of transformed *M. smegmatis* at concentrations of 44–132 mM or mitomycin C treatment of *M. smegmatis* at concentrations of 0.005–0.5 μ g ml⁻¹ produced no beneficial effect upon the level of HR, but greatly reduced cell viability (not shown).

An alternative approach for trying to boost the level of HR was altering the state of the DNA used in transformations to provide a more recombinogenic substrate, or to induce the expression of proteins involved in DNA repair mechanisms. Pretreatment of DNA by alkali denaturation, boiling or UV irradiation prior to transformation was evaluated using recombination assays.

Fig. 1. Plasmid diagrams. (a) pAGAN40, of which the region containing the *hyg* cassette is shown here, was constructed by cloning a *Bgl*I fragment containing a *hyg* cassette from pJ963 (Lydiat *et al.*, 1989) into the *Bam*HI site within the multiple cloning site of pMV206 (Stover *et al.*, 1991). The wide bar shows the cassette used throughout this work; the black arrow indicates the coding sequence. Below, the regions used in constructing pRAM4 are indicated. (b) pRAM4. Recombination assay vector, containing 517 bp duplication of the *hyg* gene surrounding the *kan* gene. The 5' *EcoRV*–*BstEII* *hyg* region and the 3' *BclI*–*PvuII* region were cloned into pMV206. The 5' and 3' regions are indicated underneath the *hyg* cassette of pAGAN40 (see above). Shading as for (a). (c) pSYCH09 suicide vector. The *hisD* gene from *M. smegmatis* was amplified as a 1.4 kb fragment and cloned into pCR11 (Invitrogen) to produce pSYCH03. The *hyg* gene from pAGAN40 (a) was excised as an *EcoRV*–*PvuII* fragment and inserted into the unique *NruI* site in the middle of *hisD*. In this diagram, the black arrow indicates the entire *hyg* cassette, including non-coding regions, while the cross-hatched bars indicate the *hisD* coding sequence. The mycobacterial DNA regions flanking the *hyg* gene were 0.6 and 0.8 kb in length. (d) p19K5 suicide vector (Mahenthiralingam *et al.*, 1998). The *M. intracellulare* 19Ag ORF, *aacC1* (gentamicin resistance, Gm^R) gene insertion and binding sites for PCR primers BIM-9 and BIM-10 are shown. The black arrow indicates the entire *aacC1* cassette, including non-coding regions, while the cross-hatched regions indicate the *M. intracellulare* DNA, the wide bar being the 19 kDa coding sequence. The mycobacterial DNA regions flanking the *aacC1* gene were 1.9 and 4.3 kb in length. (e) pHLY3 suicide vector. A 0.3 kb *EcoRV*–*SpeI* *tlyA* fragment was replaced with the *hyg* gene (*XbaI*–*SmaI*) in the same transcriptional orientation as in pCIG19 (Wren *et al.*, 1998) followed by excision of the complete 3.6 kb *SmaI* fragment and insertion into the phagemid vector pGEM3Zf(+). The black arrow indicates the entire *hyg* cassette, including non-coding regions, while the cross-hatched regions indicate the *M. tuberculosis* DNA, the wide bar being the *tlyA* coding sequence. The mycobacterial DNA regions flanking the *hyg* gene were each 1.25 kb in length. *amp*, ampicillin resistance gene; *kan*, kanamycin resistance gene; *oriE*, *E. coli* origin of replication; *hyg*, hygromycin resistance gene; *oriM*, mycobacterial origin of replication; *f1 ori*, origin for generation of ss phagemid DNA.

Fig. 2. Effect of treatment of plasmid DNA on recombination frequencies. Recombination assays were performed as described in Methods and the total number of Kan^R and Hyg^R colonies were counted. Results are expressed as the increase in numbers of recombinants (Hyg^R transformants) over the control using untreated DNA. The means $\pm$ SD of four independent experiments are shown. DNA was treated as described in the Methods.

Alkali treatment of DNA showed a marked increase in the number of recombinants (Fig. 2). While this work was in progress, Oh & Chater (1997) reported that alkali treatment of DNA improved HR in *Streptomyces coelicolor* A3(2), consistent with our results. Boiling DNA prior to transformation might have been expected to enhance recombination in the same way as alkali did, as it would also denature the DNA. However, it reduced the number of recombinants (Fig. 2), although this was largely due to lower transformation efficiency, perhaps

because the incubation was too long and caused irreparable damage to the DNA.

UV irradiation of DNA at 25 mJ cm⁻² prior to transformation slightly increased the number of recombinants and 100 mJ cm⁻² was even more effective (Fig. 2). At 300 mJ cm⁻², however, there was no increase; as with boiling, this appeared to be due to excessive DNA damage, as the total number of transformants dropped sharply (not shown).

Improvement of gene replacement frequency

It was possible that factors causing an increase in intraplasmid recombination might not be effective in improving recombination between plasmid and chromosome, which is necessary for gene replacement. Therefore, the effect of using alkali-denatured or UV-irradiated DNA on HR was assessed using the *M. smegmatis* *pyrF* gene replacement vector pY6002. Transformant numbers were increased using alkali- or UV-treated DNA (Table 2), with the alkali enhancement being statistically significant ($P < 0.01$). Transformation numbers obtained with 1 μ g untreated *E. coli* vector DNA were reduced, but not significantly, using alkali-denatured DNA; thus IR still occurred, but the relative frequency of these events decreased as the number of HR events increased. To confirm what events had taken place, colonies were tested for auxotrophy (indicating DCO events). Four out of 81 colonies tested were uracil auxotrophs. DNA from one of the auxotrophs and 11 prototrophs were analysed on Southern blots. The auxotroph had the expected DNA profile, while 8/11 of the prototrophs were SCO events and the remaining three were illegitimate. Thus in this experiment, 4.9% of transformants were DCOs, 69.2% were SCOs and 25.9% were illegitimate.

The effect of using alkali-denatured or UV-irradiated DNA on *M. smegmatis* *hisD* gene replacement was then assessed using pSYCH09 (Table 2). The number of

Table 2. Colonies produced in *M. smegmatis* following electroporation with suicide plasmids

Vector	Treatment	n*	Untreated DNA		Treated DNA	
			No. of colonies	SD	No. of colonies	SD
pSYCH09 (<i>hisD</i>)	Alkali	16	2.6	2.3	5.2	6.1
	UV	4	2.3	2.6	6.3	2.9
	ssDNA	9	5.3	8.6	1.0	1.2
pY6002 (<i>pyrF</i>)	Alkali	11	48.3	39.4	384.0†	393.0
	UV	4	18.2 $\pm$ 6.8	13.6	328.0	146.0
<i>E. coli</i> vector	Alkali	9	2.7 $\pm$ 0.7	2.0	0.8	0.8
	ssDNA	14	7.2 $\pm$ 3.5	12.9	0.0†	0.0

*Each pair within each treatment used a separate preparation of competent cells.

† $P < 0.01$ according to Wilcoxon's signed rank sum test.

Fig. 3. Southern analysis of *M. smegmatis* *hisD::hyg* transformants. Genomic DNA was isolated from *M. smegmatis* transformants generated with (a) alkali-denatured DNA and (b) ss phagemid DNA. The figure shows blots of 1 µg DNA digested with *Sma*I and hybridized with labelled 1.4 kb *hisD* probe. WT, wild-type DNA.

colonies obtained using treated DNA was not significantly different compared to untreated DNA. However, the HR rate was much better, with 7/62 (11.3%) and 3/24 (12.5%) transformants being *his* auxotrophs after alkali and UV treatment, respectively, in great contrast to untreated pSYCH09 DNA, where no auxotrophs were seen in 146 transformants. Southern blotting of six auxotrophs showed that five had a straightforward DCO (Fig. 3a, lanes 1–3, 5–6); *Sma*I cuts the disrupted gene into two bands of 2.4 and 2.7 kb, which are not clearly resolved in the figure. One of the auxotrophs (Fig. 3a, lane 4) contained an extra copy of *hisD* on a band of a size consistent with an SCO event in addition to the DCO (as if the entire 7.1 kb pSYCH09 had integrated into the disrupted gene, either by plasmid dimer formation, or after the first chromosomal integration); this explanation was supported by the fact that a similar band was seen in other tracks (see below).

Prototrophic transformants were again found to be a mixture of events generated by SCO HR and IR events. SCO events lost the wild-type band, but gained either a 2.4 or 2.7 kb band (Fig. 3a, lanes 7–8, 10–11). Two of these had an extra 7.1 kb band (lanes 8, 10), indicating the presence of a second copy of the plasmid in the *hisD* locus. The IR events left the wild-type 3.6 kb *Sma*I fragment intact and have an extra band of a different size (Fig. 3a, lanes 9, 12). Thus the use of denatured or irradiated DNA produced a marked improvement in the frequency of HR and achieved *hisD* gene inactivation.

Following the improvement achieved using denatured DNA, the effect of using ss phagemid DNA was assessed using pSYCH09 (Table 2). A striking observation was the total abolition of illegitimate events with vector DNA, which was statistically significant ($P < 0.01$). One out of 14 transformants tested was a histidine auxotroph and Southern blotting confirmed that this transformant was produced by a DCO HR event (Fig. 3b, lane 1). The

prototrophic transformants analysed were all found to have arisen from SCO HR events, with patterns identical to those obtained previously (Fig. 3b, lanes 2–12). Thus ssDNA was not a suitable template for integration by IR, but was able to participate in HR efficiently in *M. smegmatis* – an ideal substrate for gene replacement experiments.

Gene replacement in *M. intracellulare*

The effect of using denatured dsDNA as a mutagenesis template was examined in *M. intracellulare* using the gene encoding the 19 kDa lipoprotein antigen (Booth *et al.*, 1993; Prestidge *et al.*, 1995) as a model target. Successful gene replacement of this gene using a suicide vector mutagenesis strategy had been carried out in *M. intracellulare* strain 1403, with native dsDNA as the mutagenesis template (Mahenthiralingam *et al.*, 1998). The 19 kDa suicide plasmid, p19K5 (Fig. 1d), was introduced into *M. intracellulare* by electroporation in both denatured and native form. DNA recombination among the transformants was assessed using a PCR screen with primers BIM-9 and BIM-10, the binding sites of which flanked the point of 19Ag gene disruption (see Fig. 1d). Two patterns of 19Ag gene amplification were seen among transformants, a single product corresponding to the mutant allele (indicating a DCO) or two bands corresponding to the wild-type and mutant alleles (indicating either SCO or IR events). The use of denatured dsDNA increased the number of transformants ($\mu\text{g DNA}^{-1}$) fourfold (from 10 μg^{-1} for native DNA to 40 μg^{-1} for denatured DNA). In addition, the number of clones which appeared to have undergone DCO HR with the suicide vector increased from 10 to 76% of the total examined when denatured DNA was used (10/51 for native DNA; 40/50 for denatured DNA). Allelic replacement of the 19Ag gene and abrogation of protein expression was demonstrated by

Fig. 4. Southern analysis of *M. tuberculosis* *tlyA::hyg* transformants. Genomic DNA was isolated from 12 *M. tuberculosis* transformants generated using ss phagemid DNA. One microgram was digested with *Sma*I and hybridized to the *tlyA::hyg* probe (Fig. 1e). Lanes 1 and 6 show the expected pattern for a DCO, all other lanes show a pattern indicative of an SCO. The bands corresponding to the wild-type allele (WT *tlyA*), disrupted allele (*tlyA::hyg*) and plasmid vector sequences are indicated. The vector bands are indicated. These are fainter than the other bands because they are present due to contaminating vector DNA in the probe.

Southern hybridization and immunoblot, respectively, for two putative DCO transformants (Mahenthiralingam *et al.*, 1998).

Gene replacement in *M. tuberculosis*

The methodology of using UV-irradiated dsDNA and ss phagemid DNA was also applied to gene replacement in *M. tuberculosis* using a putative haemolysin-encoding gene (*tlyA*) (Wren *et al.*, 1998). Transformation with the suicide vector pHLY3 (Fig. 1e) generated 10 transformants using 5 µg UV-irradiated plasmid dsDNA and 12 transformants using 2 µg ss phagemid DNA, while no colonies were obtained using untreated DNA. Southern blotting of 20 transformants thus generated (8 from dsDNA, 12 from ss phagemid DNA) showed that all arose by HR events, with no IR being observed. The 12 transformants obtained using phagemid DNA are shown in Fig. 4; all transformants contain the 3.6 kb band containing the *tlyA::hyg* construct, while two lack the 2.4 kb wild-type *tlyA* band. All the strains with putative SCO events contain the plasmid vector, while those with putative DCO events do not (Fig. 4, lanes 1 and 6). Analysis of DNA digested with *Pvu*II (which cuts within the probe; not shown) confirmed that the other 18 transformants tested were all SCOs rather than IR events. Thus using phagemid DNA, 2/12 transformants (16%) had arisen from a DCO HR event, while the other 10 contained SCO events. Using UV-irradiated plasmid DNA, 1/8 (12%) of the transformants was produced by DCO and 7/8 by SCO HR events.

DISCUSSION

The inactivation or mutation of genes in a targeted fashion has been difficult in slow-growing mycobacteria. It is difficult to generalize from other work, because one

feature of gene replacement is that different genes behave differently; while inactivation of one gene may be obtained readily, another may prove refractory. For example, one reason for the success using *pyrF* compared to *hisD* may be the longer flanking DNA in the *pyrF* construct (2–3 kb, compared to 0.6–0.8 kb with *hisD*). However, we have used other genes in *M. smegmatis* with several kb of flanking DNA and none has been as easy to inactivate as *pyrF*, suggesting other factors are also involved. Whatever the causes of these differences, the way forward seems to be to develop methods which optimize the possibilities of obtaining a mutant. This would include increasing the rate of HR, reducing the background of spontaneous mutants or IR events, increasing the total numbers of colonies obtained and improving the selection and screening methods to reduce the number of colonies that need analysing.

We have shown that HR in *M. smegmatis* using non-replicating suicide plasmid vectors was enhanced by the use of ssDNA (either generated by alkali denaturation of a dsDNA plasmid template or by production of ss phagemid DNA), as reported for *S. coelicolor* A3(2) (Oh & Chater, 1997), or alternatively by the use of UV-irradiated dsDNA. Using these techniques, gene replacement was improved with two genes in *M. smegmatis* and with genes in two slow-growing mycobacterial species, *M. intracellulare* and *M. tuberculosis*. The improvement in HR seen may be due to stimulation of *recA* or other DNA repair systems. The stimulation of HR by UV may be caused by the production of a gapped duplex substrate following nucleotide excision repair (by UvrABC). This has been shown to be an effective recombination substrate due to the rapid formation of RecA filaments (Friedberg *et al.*, 1995). Although both alkali and UV appear to work, caution may be necessary when using UV, as there is the potential for the induction of point mutations in the recombined DNA.

IR was reported as being a serious problem in *M. tuberculosis*, but not in *M. smegmatis* (Kalpana *et al.*, 1991). We have shown that there is also a background level of IR in the fast-growing species; although this is not a major obstacle when using genes where HR is high, it can cause difficulties where HR is low. We obtained fewer background colonies using alkali, although this did not reach statistical significance. However, the use of ss phagemid DNA totally removed this problem – to date we have not seen IR in *M. smegmatis* using this technique. Furthermore, we obtained similar results in *M. intracellulare* and *M. tuberculosis*, strongly suggesting that IR need not be a problem in slow-growing mycobacteria, even if it is true that the rate of IR is higher than in fast-growing species.

Successful gene replacement has now been achieved in BCG using non-replicating circular (Norman *et al.*, 1995) or linear (Reyrat *et al.*, 1995; Azad *et al.*, 1996, 1997) DNA and in *M. tuberculosis* using long linear DNA substrates (Balasubramanian *et al.*, 1996) or a thermosensitive replicating plasmid (Pelicic *et al.*, 1997). The need to develop the ability to inactivate genes at will

is even greater, now that the genome sequence of *M. tuberculosis* has been completed (Cole *et al.*, 1998). Our methodology is very simple and does not require complex vector construction, as with the long linear substrate approach. Also, the ability to use *E. coli* plasmids may well be more practical than use of a shuttle vector with a mycobacterial origin of replication, in that finding suitable restriction sites for disruption in a particular gene can be difficult even without the extra DNA present. Pelicic *et al.* (1997) used *sacB* as a negative selectable marker and it would be possible to use this in conjunction with the system described here to select against SCOs. Another alternative would be to use selection for streptomycin sensitivity using *rpsL*⁺ (Sander *et al.*, 1995), although this requires a streptomycin-resistant host.

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