Production of mutants in amino acid biosynthesis genes of *Mycobacterium tuberculosis* by homologous recombination

Tanya Parish,¹ Bhavna G. Gordhan,² Ruth A. McAdam,³ Ken Duncan,³ Valerie Mizrahi^{2,4} and Neil G. Stoker¹

Author for correspondence: Neil G. Stoker. Tel: +44 20 7927 2425. Fax: +44 20 7637 4314.
e-mail: neil.stoker@lshtm.ac.uk

¹ Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK

² Molecular Biology Unit, South African Institute for Medical Research, PO Box 1038, Johannesburg 2000, South Africa

³ Immunopathology Unit, Glaxo Wellcome Research and Development, Medicines Research Centre, Stevenage, UK

⁴ Department of Haematology and Molecular Medicine, University of the Witwatersrand, Johannesburg, South Africa

The ability to generate mutants of *Mycobacterium tuberculosis* will be important if we are to understand the biology of this major pathogen. However, allelic replacement methods have only recently achieved success. We have developed a reproducible method for generating defined mutants of *M. tuberculosis* using homologous recombination. The transforming DNA was used following pre-treatment either with UV light or alkali denaturation in order to stimulate homologous recombination and abolish illegitimate recombination. Suicide vectors carrying one of nine amino acid biosynthesis genes were electroporated into *M. tuberculosis*, and homologous recombinants were obtained in all nine genes; eight resulted from single-crossover events (SCOs) and one from a double-crossover event (DCO) (in the *metB* gene). The remaining colonies were spontaneous hygromycin-resistant mutants; no products of illegitimate recombination were observed. To more efficiently distinguish spontaneous mutants, the *lacZ* gene was cloned into five vectors (two containing genes not previously tested), and the transformations were repeated. SCO mutants were identified by screening for blue colonies on indicator plates. White transformants were tested for auxotrophy and *trpD*, *hisD* and *proC* auxotrophic mutants were obtained. Only blue SCOs were obtained for *argF* and *glnE*. Thus, using this methodology we have obtained homologous recombination in 11 genes, and DCOs in 4 genes, showing that it is possible to generate targeted mutants in a reproducible way.

Keywords: auxotrophs, gene replacement, *lacZ*, Casamino acids, pre-treated DNA

INTRODUCTION

An understanding of the genetics of *Mycobacterium tuberculosis* is important both for characterization of potential drug targets and for a deeper knowledge of the mechanisms by which the bacterium establishes an infection and causes disease. The ability to inactivate genes at will is a key requirement for this, made even more desirable by the data which are now available following the completion of the genome sequence (Cole *et al.*, 1998). Gene replacement has not proved to be straightforward in mycobacteria, and has only recently been achieved in *M. tuberculosis* (Balasubramanian *et*

al., 1996; Hinds *et al.*, 1999; Pavelka *et al.*, 1999; Pelicic *et al.*, 1997). Illegitimate recombination (IR) has been demonstrated in *M. tuberculosis* and appears to occur at a relatively high rate (Kalpana *et al.*, 1991). Early reports suggested that IR did not occur in *Mycobacterium smegmatis*, but we have shown that IR also occurs in this fast-growing species at a low level (Hinds *et al.*, 1999). Where the rate of IR exceeds that of homologous recombination (HR), the isolation of knockout mutants becomes a problem.

We have previously shown that pre-treatment of the transforming DNA increases the rate of HR, which enabled us to isolate mutants in three mycobacterial species: *M. smegmatis*, *Mycobacterium intracellulare* and *M. tuberculosis* (Hinds *et al.*, 1999). Although we had obtained a *tlyA* mutant of *M. tuberculosis* using

Abbreviations: DCO, double crossover; HR, homologous recombination; IR, illegitimate recombination; hyg^R, hygromycin resistant; SCO, single crossover.

pre-treated DNA (Hinds *et al.*, 1999), it was important to look at different genes in order to assess the general applicability of this delivery system. Here we describe the use of DNA pre-treatment to test our system with a further 11 genes and present an improved procedure for simply and reliably isolating targeted mutants of *M. tuberculosis*. We selected genes involved in amino acid biosynthesis as our candidates for knockouts because mutants in these genes may be attenuated *in vivo* as has been documented for *Salmonella typhimurium* (O'Callaghan *et al.*, 1988). Such mutants would provide important information regarding the host environment encountered by the bacterium and may be potentially useful in new vaccine development. The additional advantages were that mutants would have easily testable phenotypes and the genes are unlikely to be essential on rich media *in vitro*.

METHODS

Bacterial strains and plasmids. Plasmids used in this study are listed in Table 1. Middlebrook 7H9 broth and Middlebrook 7H10 agar (Difco) plus 10% OADC supplement (Becton Dickinson) and 0.05% Tween 80 for liquid media were used for culture of *M. tuberculosis*. Hygromycin was added to 50–100 µg ml⁻¹ and X-Gal at 50 µg ml⁻¹ where appropriate. Filter sterilized Casamino acids were used at 40 µg ml⁻¹ and individual amino acids were used at 50 µg ml⁻¹. 'Aro' supplement consisted of the three amino acids L-tryptophan, L-phenylalanine and L-tyrosine at 50 µg ml⁻¹ each and *p*-aminobenzoic acid at 100 µg ml⁻¹.

Construction of delivery vectors. Cosmids were obtained from the Sanger Centre and restriction fragments were subcloned into pGEM3Zf(+), pGEM5Zf(+) or pUC18 by standard methods (Sambrook *et al.*, 1989). The hygromycin resistance gene (*hyg*) from pAGAN40 (Hinds *et al.*, 1999) was then cloned into each gene.

Construction of *lacZ* delivery vectors. The complete

Escherichia coli lacZ gene from plasmid pATB12 (Martin Everett, Glaxo Wellcome) was cloned as a *Hind*III fragment into the *Hind*III site of five suicide vectors (Table 2) and expression of *lacZ* confirmed in *E. coli* as demonstrated by the formation of blue colonies on X-Gal plates. The *lac* promoter is not itself functional in mycobacteria, so we assume that *lacZ* expression in mycobacteria occurs from a cryptic promoter.

Electroporation of *M. tuberculosis* H37Rv. Liquid cultures (10 ml) were inoculated from plates and cultured standing for 4–6 weeks at 37 °C. One millilitre of this culture was used to inoculate a further 10 ml and used as the seed culture. One hundred millilitre cultures were inoculated with 1–10 ml of seed culture and grown in a roller bottle for 4–7 d. Glycine was added to 0.2 M 24–72 h prior to harvesting. Cultures were harvested, washed in 1/10 vol 10% glycerol (prewarmed to 37 °C), then 1/20 vol and finally resuspended in 1–2 ml 10% glycerol. Pre-treated DNA (1–5 µg) was added to 200 µl cell suspension and subjected to a pulse of 2.5 kV, 25 µF, 700 Ω using a Bio-Rad Gene Pulser at room temperature. Cells were recovered into 10 ml 7H9/OADC/Tween containing supplements as required and incubated at 37 °C for 3–24 h before plating onto 7H10/OADC plus appropriate antibiotics and supplements. Plates were incubated at 37 °C for 3 weeks.

Treatment of transforming DNA. Vector DNA was treated prior to transformation as described (Hinds *et al.*, 1999). Briefly, UV irradiation of DNA was carried out in an UV Stratalinker 1800 (Stratagene) at 100 mJ cm⁻². Alkali denaturation was performed in a 100 µl volume containing 0.2 M NaOH, 0.2 mM EDTA at 37 °C for 30 min, followed by ethanol precipitation.

Analysis of transformants. Transformants were inoculated into 10 ml liquid media (plus hygromycin and supplements) and cultured standing at 37 °C for DNA preparation by the method of Belisle & Sonnenberg (1998) or Santos *et al.* (1992). Southern blotting was carried out using probes labelled by the AlkPhos Direct method (Amersham). Initial auxotrophy testing was carried out by patching transformant colonies onto 7H10/OADC plates with and without the appropriate supplements and cultured at 37 °C for 3–4 weeks before

Table 1. Plasmids used in this study

Plasmids	Description	Source/reference
pGEM3Zf(+)	Phagemid vector, Ap ^R	Promega
pGEM5Zf(+)	Phagemid vector, Ap ^R	Promega
pUC18	Cloning vector, Ap ^R	Stratagene
pAGAN40	<i>E. coli</i> –mycobacteria shuttle vector, <i>hyg</i>	Hinds <i>et al.</i> (1999)
pARG7	<i>argF</i> :: <i>hyg</i> suicide delivery vector, <i>lacZ</i>	This work
pAROB2	<i>aroK</i> :: <i>hyg</i> suicide delivery vector	This work
pGLN2	<i>glnE</i> :: <i>hyg</i> suicide delivery vector	This work
pGLN7	<i>glnE</i> :: <i>hyg</i> suicide delivery vector, <i>lacZ</i>	This work
pHIS2	<i>hisD</i> :: <i>hyg</i> suicide delivery vector	This work
pHIS7	<i>hisD</i> :: <i>hyg</i> suicide delivery vector, <i>lacZ</i>	This work
pSELF23	<i>metB</i> :: <i>hyg</i> suicide delivery vector	This work
pLYS3	<i>lysA</i> :: <i>hyg</i> suicide delivery vector	This work
pPHE2	<i>pheA</i> :: <i>hyg</i> suicide delivery vector	This work
pPRO7	<i>proC</i> :: <i>hyg</i> suicide delivery vector, <i>lacZ</i>	This work
pTHR4	<i>thrC</i> :: <i>hyg</i> suicide delivery vector	This work
pTHR5	<i>thrB</i> :: <i>hyg</i> suicide delivery vector	This work
pTRP2	<i>trpD</i> :: <i>hyg</i> suicide delivery vector	This work
pTRP7	<i>trpD</i> :: <i>hyg</i> suicide delivery vector, <i>lacZ</i>	This work

Table 2. Construction of suicide delivery vectors

Plasmid	Gene	Rv ORF no.	Cosmid*	Fragment	Size (bp)	Vector used	Type of mutation†
pARG7	<i>argF</i>	1656	6H11	<i>Bam</i> HI– <i>Bgl</i> II	3812	pGEM3Zf(+)	156 bp deletion
pAROB2	<i>aroK</i>	2539c	Y159	<i>Sph</i> I	2780	pGEM5Zf(+)	324 bp deletion
pGLN2	<i>glnE</i>	2221c	Y190	<i>Bam</i> HI– <i>Bgl</i> II	6552	pGEM3Zf(+)	1679 bp deletion
pHIS2	<i>hisD</i>	1599	Y223	<i>Xho</i> I– <i>Eco</i> RI	3525	pGEM3Zf(+)	68 bp deletion
pLYS3	<i>lysA</i>	1293	Y373	<i>Pst</i> I– <i>Sma</i> I	3025	pGEM5Zf(+)	Insertion
pSELF23	<i>metB</i>	1079	Y75	<i>Pst</i> I– <i>Eco</i> RI	3608	pUC18	Insertion
pPHE2	<i>pheA</i>	3838c	1A6	<i>Pvu</i> II	4322	pGEM5Zf(+)	361 bp deletion
pPRO7	<i>proC</i>	500	20G9	<i>Bam</i> HI– <i>Eco</i> RI	4869	pGEM3	198 bp deletion
pTHR5	<i>thrB</i>	1296	Y373	<i>Kpn</i> I– <i>Pst</i> I	3041	pGEM5Zf(+)	Insertion
pTHR4	<i>thrC</i>	1295	Y373	<i>Kpn</i> I– <i>Pst</i> I	3041	pGEM5Zf(+)	Insertion
pTRP2	<i>trpD</i>	2192c	Y190	<i>Eco</i> RI	5362	pGEM3Zf(+)	327 bp deletion

* Cosmids were obtained from the Sanger Centre and the indicated fragments subcloned into an *E. coli* cloning vector.

† The *hyg* gene from pAGAN40 (Hinds *et al.*, 1999) was cloned into the indicated genes either as a straightforward insertion or by replacing part of the gene.

reading results. Auxotrophy was confirmed both by plating liquid cultures onto 7H10/OADC plates with and without individual amino acid supplements and by subculturing into unsupplemented 7H9/OADC/Tween broth and checking for lack of growth.

RESULTS

Isolation of single crossover (SCO) homologous recombinants in amino acid biosynthesis genes

The *M. tuberculosis* genome sequence (Cole *et al.*, 1998) was used to select nine amino acid biosynthesis genes (Table 2), and knockout constructs were made in suicide

plasmid vectors (plasmids that can replicate in *E. coli*, but not in mycobacteria). This was achieved by cloning each of the *M. tuberculosis* genes and inserting the *hyg* gene either as a simple insertion or by replacing a fragment of the gene (Table 2). DNA samples from nine plasmids containing disrupted genes for *aroK*, *glnE*, *hisD*, *lysA*, *metB*, *pheA*, *thrB*, *thrC* and *trpD* were pre-treated with UV or alkali and used to transform *M. tuberculosis*. The cells were plated onto 7H10/OADC medium supplemented with Casamino acids, and hygromycin-resistant (*hyg*^R) transformants were obtained. Table 3 shows the total numbers of transformants obtained from one to four independent electroporations

Table 3. Analysis of transformants using pre-treated suicide vector DNA

Vector DNAs were pre-treated as described in Methods and used to electroporate *M. tuberculosis*. *Hyg*^R transformants were selected on 7H10/OADC supplemented with Casamino acids, and Southern blotting was used to determine the genotype of each transformant. The figures shown are the totals from 1 to 4 electroporations per gene. The electroporation efficiencies with the replicating plasmid pATB12 varied from 10⁴ to 10⁶ (µg DNA)^{−1}.

Gene	Alkali		UV	
	Spontaneous mutants	Homologous recombination	Spontaneous mutants	Homologous recombination
<i>aroK</i>	8	0	13	1
<i>glnE</i>	1	0	6	6
<i>hisD</i>	0	0	2	2
<i>lysA</i>	5	0	13	2
<i>metB</i>	4	0	0	1*
<i>pheA</i>	0	0	2	1
<i>thrB</i>	1	0	2	2
<i>thrC</i>	0	0	5	9
<i>trpD</i>	2	1	11	11

* This was the only recombinant in which a DCO had taken place; all others were SCOs.

Table 4. Analysis of transformants using pre-treated *lacZ*-suicide vector DNA

Vector DNAs were pre-treated as described in Methods and used to electroporate *M. tuberculosis*. Hyg^R transformants were selected on 7H10/OADC medium supplemented with individual amino acids and X-Gal to score for blue and white colonies. The genetic structures of several blue colonies were confirmed as SCOs by Southern analysis. White colonies were streaked onto 7H10/OADC plates with or without amino acid supplement for auxotrophy testing. The figures shown are the totals from 2 to 5 electroporations per gene.

Gene	Alkali			UV		
	Blue	White	Auxotrophs*	Blue	White	Auxotrophs*
<i>argF</i>	36	11	0	25	1	0
<i>glnE</i>	100	19	0	400	8	0
<i>hisD</i>	7	2	0	41	15	12
<i>proC</i>	49	12	0	63	16	2
<i>trpD</i>	16	1	1	115	35	35

* All white colonies were tested for auxotrophy except with *proC*, where only 8 alkali-generated colonies and 7 UV-generated colonies were tested.

per gene. Transformants were grown up, genomic DNA was isolated, and Southern blotting was used to classify them as SCOs, double crossovers (DCOs), products of IR or spontaneous mutants (data not shown). Analysis of all 111 transformants showed that 35 were SCOs, one was a DCO, 75 were spontaneous hyg^R mutants and none resulted from IR. Homologous recombinants were obtained at all nine loci. A DCO was obtained in the *metB* gene only, with only SCOs occurring at the remaining loci.

Another group had found that Casamino acids are toxic to auxotrophs (Pavelka & Jacobs, 1999). Our results were consistent with this observation, as we had only obtained a DCO mutant in one of the amino acid biosynthesis genes tested, and this one (*metB*) was actually prototrophic. Therefore, in later experiments we plated transformations onto media containing the appropriate amino acid as a supplement rather than Casamino acids.

Isolation of DCOs using *lacZ*

The large number of spontaneous hyg^R mutants obtained prompted us to include the *lacZ* gene from *E. coli* as a screenable marker in the suicide vectors. This would enable us to identify SCOs in the initial transformation since they would form blue colonies on plates containing X-Gal and hygromycin; white colonies should arise from DCO events (as well as spontaneous hygromycin resistance). The *lacZ* gene was therefore cloned into three vectors already used (containing disrupted *trpD*, *hisD* and *glnE* genes) and two new suicide vectors were constructed, containing disrupted *proC* and *argF* genes (Table 2). DNA was pre-treated with either UV or alkali and used in electroporations.

Results from several experiments are shown in Table 4.

Table 5. Flanking DNA and sites of HR

SCO transformants were analysed by Southern blotting and where possible the SCO structure was determined to see on which side HR had occurred. Flanking DNA length (bp) on each side of the *hyg* gene is given along with the site of HR. ND, not determined.

Gene	Flanking DNA		Site of HR
	5'	3'	
<i>aroK</i>	954	1502	3'
<i>glnE</i>	3775	1098	5'
<i>hisD</i>	1210	2247	3'
<i>lysA</i>	1677	1348	ND
<i>metB</i>	2867	745	Both
<i>pheA</i>	2865	1092	ND
<i>thrC</i>	440	2601	3'
<i>thrB</i>	1452	1589	3'
<i>trpD</i>	1783	3251	3'

The total number of transformants was much higher than in the previous experiments, but the numbers were still very variable between experiments. Furthermore, the number of transformants obtained (and the ability to isolate recombinants) did not appear to correlate with the electroporation efficiencies obtained using replicating plasmids. For example, with the *glnE* plasmid, on one occasion we obtained 200 blue and 2 white colonies (10^4 c.f.u. per μ g replicating plasmid) and on another we obtained only 3 blue and 11 white colonies (10^5 c.f.u. per μ g replicating plasmid). We do not know what factors are responsible for this variation.

The many blue colonies obtained suggested that SCOs

had taken place, and several clones were tested by Southern blotting to confirm this conclusion (data not shown). Since white colonies could have arisen either from spontaneous hygromycin resistance or from a DCO event, they were patch-tested for amino acid requirement. Several auxotrophs were isolated, occurring in three different genes: *hisD*, *proC* and *trpD*. Auxotrophy was confirmed in liquid culture, and Southern blotting of three *hisD*, two *proC* and three *trpD* auxotrophic mutants confirmed the expected DCO genotype. Thus, in these cases, the *lacZ* marker was useful since it showed that HR was taking place, as evidenced by the formation of SCOs, and allowed us to focus on the white colonies in screening for DCOs.

For *glnE*, a large number of blue colonies (500) from three experiments, but very few whites, were isolated. All of the white colonies were found to be spontaneous *hyg^R* mutants; the absence of any DCOs may indicate that this gene is essential for growth on the medium used. The use of *lacZ* enables one to determine the frequency at which HR is taking place to produce SCOs, thus allowing a quick preliminary screen for essential genes – the absence of DCOs in such a large population of SCOs is indicative of HR, but suggests that DCOs may be lethal under the conditions used. No *argF* mutants were isolated; the number of blue colonies was lower than that obtained with *glnE*, so there was less compelling evidence that a DCO would be deleterious in this case.

DISCUSSION

M. tuberculosis mutants

Four defined mutants have been constructed in amino acid biosynthesis genes by a method that uses non-replicating DNA pre-treated with UV or alkali. Auxotrophic mutants are often attenuated (Cersini *et al.*, 1998; Moral *et al.*, 1998; Valentine *et al.*, 1998), presumably because the bacteria live in an environment lacking that particular amino acid at some point during infection. Guleria *et al.* (1996) showed that *M. bovis* BCG auxotrophic for methionine, leucine or isoleucine-leucine-valine are attenuated in mice. The mutants described in this paper are currently being tested for their ability to grow in macrophage/mouse models. As well as informing us about the *in vivo* environment, rationally attenuated strains can also be used as the basis for new vaccine production. They would also be useful general genetic tools, for example as markers, for the production of safer strains for genetic manipulation or for labelling of cell protein.

The *metB* mutant obtained was not auxotrophic, showing no growth requirement for methionine. This gene encodes a putative cystathionine γ -synthase, which converts *O*-succinyl-L-homoserine to cystathionine. One possible explanation is the possession of another gene/enzyme, that can complement or compensate for the loss of MetB activity. The complete genome sequence reveals that another gene (*metZ*; Rv0391) may also be a

cystathionine γ -synthase, so it is possible that this gene product may have an overlapping function. Alternatively, the site of the *hyg* gene insertion may not have inactivated the gene function, although the insertion lies near the 5' end (70 bp downstream of the predicted start codon).

Initial attempts to construct defined auxotrophs by HR were unsuccessful. Only one DCO mutant was obtained and that was prototrophic. The *hisD* auxotroph was tested for its ability to grow on media supplemented with Casamino acids and failed to grow, confirming that they are toxic at the concentration we used (0.5%). This confirms the recent observations of Pavelka & Jacobs (1999), and is an important consideration for other groups trying to isolate auxotrophs.

In the case of the SCOs analysed, the side on which recombination had occurred was that on which the flanking DNA was longer. The size ranged from 1.5 kb (*aroK*) to 3.8 kb (*glnE*). For the four DCOs where HR had necessarily occurred on both sides, the range of flanking DNA was from 0.7 kb (*metB*) to 3.3 kb (*trpD*). Therefore, the need for regions of homology >1kb on each side of the mutated gene is not an absolute requirement, but is probably desirable. However, there is a trade-off in using longer flanking DNA to increase the efficiency of recombination since the efficiency of transformation is expected to fall with increasing plasmid size.

IR and spontaneous mutants

IR was not observed in any of these experiments. Care was taken to ensure that the restriction enzymes and probes used for Southern blotting would distinguish SCOs from illegitimate recombinants. IR has been one of the barriers to routinely obtaining gene knockouts, since it masks the low frequency of HR and requires that significant numbers of transformants are analysed in order to find the HR transformants which, for *M. tuberculosis*, is a laborious and time-consuming process. IR has been reported in *M. tuberculosis* (Kalpana *et al.*, 1991; Pavelka & Jacobs, 1999), *M. bovis* (Wilson *et al.*, 1997) and *M. bovis* BCG (Aldovini *et al.*, 1993; Kalpana *et al.*, 1991), but now generally appears to be a nuisance rather than an overwhelming problem. The success of our method in attaining HR in 11 genes without any IR may be due to the fact that IR is not as big a problem as originally feared or that the method we use favours HR over IR, or it may be a combination of these factors.

Spontaneous resistance to hygromycin is potentially a problem because *M. tuberculosis* only has a single rRNA operon, and therefore is prone to resistance due to chromosomal mutations. Although the spontaneous mutation rate was reasonably constant, considerable variation in the efficiency of recombination for a given suicide vector was observed between transformations. As a result, the magnitude of the background problem caused by spontaneous mutants varied between experiments. However, this problem was largely overcome by

including a second screenable marker, *lacZ*, in the vector.

Differences in DNA pre-treatment

UV pre-treatment was the more successful and technically easiest method, generating larger numbers of HR transformants than alkali pre-treatment. However, a potential drawback of UV treatment is that recombination with a DNA-damaged vector might result in the inadvertent introduction of secondary mutations. Although we have not directly tested for this, the fact that no *lacZ* mutants were identified amongst the large number of SCOs characterized in this study argues against mutagenesis of the integrated DNA occurring at a high frequency. Little, if anything, is known about the mutagenesis pathways that may operate in *M. tuberculosis* in response to DNA damage. A major mechanism for UV-induced mutagenesis in *E. coli* is via the *umuCD*-dependent SOS-inducible pathway. No *umuD* has been identified in the *M. tuberculosis* genome (Cole *et al.*, 1998; Mizrahi & Andersen, 1998), suggesting that the SOS-inducible mutagenesis pathway is unlikely to be operative in this organism, although we cannot exclude the possibility that other inducible pathways exist (Humayun, 1998).

Alkali treatment was also successful in obtaining both SCO and DCO mutants, albeit at a lower rate. The difference in numbers of recombinants may reflect loss of DNA during precipitation or differences in the DNA substrates participating in the recombination events *in vivo*.

Use of *lacZ*

The inclusion of *lacZ* into the suicide vector was an effective way of revealing colonies carrying plasmids integrated by SCOs. Thus, with *lacZ* it is possible to judge firstly if the experiment looks successful in terms of achieving any HR, and secondly, allows blue colonies to be ignored when looking for DCOs. If no DCOs are found, a blue (SCO) colony can be picked and plated for a second recombination event, although the inclusion of a negative selection marker such as *sacB* may be necessary (Pelicic *et al.*, 1996).

Conclusion

We have developed a simple method for generating defined mutants of *M. tuberculosis* by HR and have demonstrated its general utility in this paper using 11 genes. This has allowed the isolation of four DCO mutants in amino acid biosynthesis genes.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of the Glaxo Wellcome Action TB Initiative, the South African Medical Research Council and the South African Institute for

Medical Research. We thank Selwyn Quan and Jason Hinds for helpful discussions, and the Sanger Centre for providing the cosmid.

REFERENCES

- Aldovini, A., Husson, R. N. & Young, R. A. (1993). The *uraA* locus and homologous recombination in *Mycobacterium bovis* BCG. *J Bacteriol* **175**, 7282–7289.
- Balasubramanian, V., Pavelka, M. S., Jr, Bardarov, S. S., Martin, J., Weisbrod, T. R., McAdam, R. A., Bloom, B. R. & Jacobs, W. R., Jr (1996). Allelic exchange in *Mycobacterium tuberculosis* with long linear recombination substrates. *J Bacteriol* **178**, 273–279.
- Belisle, J. T. & Sonnenberg, M. G. (1998). Isolation of genomic DNA from mycobacteria. In *Mycobacteria Protocols*, pp. 31–44. Edited by T. Parish & N. G. Stoker. Totowa, NJ: Humana Press.
- Cersini, A., Salvia, A. M. & Bernardini, M. L. (1998). Intracellular multiplication and virulence of *Shigella flexneri* auxotrophic mutants. *Infect Immun* **66**, 549–557.
- Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- Guleria, I., Teitelbaum, R., McAdam, R. A., Kalpana, G., Jacobs, W. R., Jr & Bloom, B. R. (1996). Auxotrophic vaccines for tuberculosis. *Nature Med* **2**, 334–337.
- Hinds, J., Mahenthalingam, E., Kempell, K. E., Duncan, K., Stokes, R. W., Parish, T. & Stoker, N. G. (1999). Enhanced gene replacement in mycobacteria. *Microbiology* **145**, 519–527.
- Humayun, M. Z. (1998). SOS and Mayday: multiple inducible mutagenic pathways in *Escherichia coli*. *Mol Microbiol* **30**, 905–910.
- Kalpana, G. V., Bloom, B. R. & Jacobs, W. R., Jr (1991). Insertional mutagenesis and illegitimate recombination in mycobacteria. *Proc Natl Acad Sci USA* **88**, 5433–5437.
- Mizrahi, V. & Andersen, S. J. (1998). DNA repair in *Mycobacterium tuberculosis*: what have we learnt from the genome sequence? *Mol Microbiol* **29**, 1331–1339.
- Moral, C. H., del Castillo, E. F., Fierro, P. L., Cortes, A. V., Castillo, J. A., Soriano, A. C., Salazar, M. S., Peralta, B. R. & Carrasco, G. N. (1998). Molecular characterization of the *Aeromonas hydrophila* *aroA* gene and potential use of an auxotrophic *aroA* mutant as a live attenuated vaccine. *Infect Immun* **66**, 1813–1821.
- O'Callaghan, D., Maskell, D., Liew, F. Y., Easmon, C. S. & Dougan, G. (1988). Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attention, persistence, and ability to induce protective immunity in BALB/c mice. *Infect Immun* **56**, 419–423.
- Pavelka, M. S., Jr & Jacobs, W. R., Jr (1999). Comparison of the construction of unmarked deletion mutations in *Mycobacterium smegmatis*, *Mycobacterium bovis* Bacillus Calmette-Guerin, and *Mycobacterium tuberculosis* H37Rv by allelic exchange. *J Bacteriol* **181**, 4780–4789.
- Pelicic, V., Reyat, J. M. & Gicquel, B. (1996). Expression of the *Bacillus subtilis* *sacB* gene confers sucrose sensitivity on mycobacteria. *J Bacteriol* **178**, 1197–1199.
- Pelicic, V., Jackson, M., Reyat, J. M., Jacobs, W. R., Jr, Gicquel, B. & Guilhot, C. (1997). Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **94**, 10955–10960.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Santos, A. R., Demiranda, A. B., Lima, L. M., Suffys, P. N. & Degrave, W. M. (1992). Method for high yield preparation in large and small scale of nucleic acids from mycobacteria. *J Microbiol Methods* **15**, 83–94.

Valentine, P. J., Devore, B. P. & Heffron, F. (1998). Identification of three highly attenuated *Salmonella typhimurium* mutants that are more immunogenic and protective in mice than a prototypical *aroA* mutant. *Infect Immun* **66**, 3378–3383.

Wilson, T., Wards, B. J., White, S. J., Skou, B., de Lisle, G. W. & Collins, D. M. (1997). Production of avirulent *Mycobacterium bovis* strains by illegitimate recombination with deoxyribonucleic acid fragments containing an interrupted *ahpC* gene. *Tuber Lung Dis* **78**, 229–235.

Received 3 June 1999; accepted 20 August 1999.