Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis* *tlyA plcABC* mutant by gene replacement

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Progress in the field of mycobacterial research has been hindered by the inability to readily generate defined mutant strains of the slow-growing mycobacteria to investigate the function of specific genes. An efficient method is described that has been used to generate several mutants, including the first double unmarked deletion strain of *Mycobacterium tuberculosis*. Four mutants were constructed: a marked deletion of the *plcABC* cluster, which encodes three phospholipases C; separate unmarked deletions in *plcABC* and *tlyA* (encoding a haemolysin); and a double unmarked mutant *tlyAΔ plcABCΔ*. To accomplish this, two series of vectors were designed, the first of which, named pNIL, allows manipulation of the target gene sequence at a variety of convenient restriction sites. The second series, named pGOAL, contains marker cassettes flanked by *PacI* restriction enzyme sites. The final suicide plasmid vectors were then obtained by cloning a marker cassette from a pGOAL vector into the single *PacI* site of the pNIL vector with the modified gene of interest. Finally, a two-step strategy was employed whereby single cross-over events were first selected, then screening for the second cross-over was carried out to yield the mutant strains. This technique will now allow the construction of potential vaccine strains without the inclusion of antibiotic resistance markers, the ability to make multiple defined mutations and the possibility of making more subtle defined mutations, such as point mutations.

Keywords: homologous recombination, haemolysin, phospholipase C, rapid cloning system

INTRODUCTION

Tuberculosis remains a serious public health problem in many parts of the world and is responsible for approximately 2 million deaths per year (Dye *et al.*, 1999). New drugs and vaccines are required to control the disease. However, progress has until recently been hampered by the lack of suitable tools with which to manipulate the genome of the causative pathogen, *Mycobacterium tuberculosis*. In particular, it has not been possible to generate defined mutants of *M. tuberculosis* by homologous recombination routinely despite early successes (Balasubramanian *et al.*, 1996; Berthet *et al.*, 1998; Pelicic *et al.*, 1997). By making such mutants, the function of individual genes may be analysed, revealing potential targets for novel chemotherapies. Further-

more, now that the completed genome sequence is available (Cole *et al.*, 1998), rationally attenuated strains which are potential vaccine candidates can be constructed.

For these reasons, we have been developing an efficient system for introducing specific mutations in *M. tuberculosis*. We have previously reported the construction of five *M. tuberculosis* strains with defined mutations in genes encoding a haemolysin (*tlyA*) (Hinds *et al.*, 1999) and enzymes from amino acid biosynthesis pathways (*hisD*, *metB*, *proC* and *trpD*) (Parish *et al.*, 1999). Our method uses a suicide plasmid vector to deliver the recombination substrate to the recipient cell. The plasmids lack a mycobacterial origin of replication (*oriM*) and are thus unable to replicate in *M. tuberculosis*. Plasmid DNA is pretreated with UV light or alkali to stimulate homologous recombination and abolish illegitimate recombination in the recipient cells. Construction of the suicide delivery vector has often

Abbreviations: hyg, hygromycin; kan, kanamycin; suc, sucrose; MCS, multiple cloning site.

been problematic as several cloning steps are required to include the relevant markers and finding appropriate restriction sites for inserting these genes has become limiting. In this paper, we describe two series of vectors that we developed to overcome the cloning bottleneck. By separating the construction of the mutated version of the target gene from that of the inclusion of the required marker genes, vector construction is rapid and flexible.

The genes that we chose for this study encode a haemolysin (*tlyA*) and three phospholipases C found in a cluster (*plcABC*) on the *M. tuberculosis* chromosome. Homologues of these genes have been shown to be important for virulence in other organisms (Hyatt *et al.*, 1994; Songer, 1997; Titball, 1993). To study the role of these genes in virulence, we selected them as candidates for the construction of isogenic mutant strains of *M. tuberculosis* H37Rv. We have demonstrated the utility of the pNIL and pGOAL cloning vectors in *M. tuberculosis* by generating both marked and unmarked deletions of the *plcABC* gene cluster and an unmarked deletion in the *tlyA* gene. The *tlyA* mutant was then used to generate a double unmarked mutant when the *plcABC* gene cluster was deleted in this strain.

METHODS

Bacterial strains and plasmids. Plasmids and strains used in this study are listed in Tables 1 and 2. *M. tuberculosis* H37Rv

Table 2. Marker gene cassettes available in the pGOAL series

pGOAL no.	Marker genes
7	<i>lacZ</i> *, <i>hyg</i>
11	<i>lacZ</i> *, <i>hyg</i> , <i>sacB</i>
12	<i>lacZ</i> *, <i>sacB</i>
13	<i>lacZ</i> *, P _{hsp60} - <i>sacB</i>
14	P _{hsp60} - <i>sacB</i>
15	<i>hyg</i> , <i>lacZ</i> *, P _{hsp60} - <i>sacB</i>
17	P _{Ag85} - <i>lacZ</i> , P _{hsp60} - <i>sacB</i>
19	<i>hyg</i> , P _{Ag85} - <i>lacZ</i> , P _{hsp60} - <i>sacB</i>

* Not expressed in *M. tuberculosis*.

was grown in Middlebrook 7H9 liquid medium or on 7H10 agar plates supplemented with 10% (v/v) OADC (Becton Dickinson) plus 0.05% (w/v) Tween 80 for liquid cultures. Kanamycin (kan) was used at 20 µg ml⁻¹, hygromycin (hyg) at 100 µg ml⁻¹, X-Gal at 50 µg ml⁻¹ and sucrose (suc) at 2% (w/v).

Construction of cloning vectors. DNA manipulations were carried out according to standard techniques (Sambrook *et al.*, 1989). The pNIL vectors were derived from pJBS633 which contains no *lac* sequences (Broome-Smith & Spratt, 1986): the 1.9 kb *EcoRV*-*PvuII* fragment was deleted and the 450 bp *EcoRI* fragment, from Tropist4, containing two *PacI* sites,

Table 1. Plasmids used

Vector	Features	Source
pBR322	Cloning vector	Broome-Smith & Spratt (1986)
pJBS633	Cloning vector	
Tropist4	Cosmid vector containing MCS with two <i>PacI</i> sites	
pEM37	P _{Ag85} - <i>lacZ</i> in <i>Mycobacterium</i> - <i>E. coli</i> shuttle vector	Edith Machowski, Johannesburg, South Africa
pATB10	<i>lacZ</i> in <i>Mycobacterium</i> - <i>E. coli</i> shuttle vector	Martin Everett, Glaxo Wellcome Stevenage, UK
p6.11	P _{hsp60} - <i>sacB</i> in <i>Mycobacterium</i> - <i>E. coli</i> shuttle vector	Selwyn Quan, Glaxo Wellcome, Stevenage, UK
p1NIL	Gene manipulation vector, <i>kan</i>	This study
p2NIL	Gene manipulation vector, <i>kan</i>	This study
pGOAL13	<i>lacZ</i> P _{hsp60} - <i>sacB</i> <i>PacI</i> cassette vector, <i>amp</i>	This study
pGOAL15	<i>hyg lacZ</i> P _{hsp60} - <i>sacB</i> <i>PacI</i> cassette vector, <i>amp</i>	This study
pGOAL17	P _{Ag85} - <i>lacZ</i> P _{hsp60} - <i>sacB</i> <i>PacI</i> cassette vector, <i>amp</i>	This study
pGOAL19	<i>hyg</i> P _{Ag85} - <i>lacZ</i> P _{hsp60} - <i>sacB</i> <i>PacI</i> cassette vector, <i>amp</i>	This study
pCIG19	2.5 kb <i>SmaI</i> fragment containing <i>M. tuberculosis</i> <i>tlyA</i> gene	Wren <i>et al.</i> (1998)
pTLY5	<i>tlyA</i> delivery vector with pGOAL15 <i>PacI</i> cassette (<i>hyg lacZ</i> P _{hsp60} - <i>sacB</i>), <i>kan</i>	This study
pPLC9	<i>plcABCΔ</i> delivery vector with pGOAL15 <i>PacI</i> cassette (<i>hyg lacZ</i> P _{hsp60} - <i>sacB</i>), <i>kan</i>	This study
pPLC10	<i>plcABCΔ::hyg</i> delivery vector with pGOAL13 <i>PacI</i> cassette (<i>lacZ</i> P _{hsp60} - <i>sacB</i>), <i>kan</i>	This study
pPLC11	<i>plcABCΔ</i> delivery vector with pGOAL19 <i>PacI</i> cassette (<i>hyg</i> P _{Ag85} - <i>lacZ</i> P _{hsp60} - <i>sacB</i>), <i>kan</i>	This study

Fig. 1. General features of the pNIL and pGOAL vector series. (a) p1NIL and p2NIL cloning vectors. Each vector carries the *kan* gene for selection in both *E. coli* and mycobacteria, an *oriE* for replication in *E. coli*, the *f1* origin for generation of phagemid ssDNA if required, a small MCS and a single *PacI* site. (b) pGOAL vector series. pGOAL vectors carry different combinations of marker genes flanked by two *PacI* sites. Each vector also carries an *oriE* and *amp* for selection in *E. coli*.

was inserted in either orientation. Finally, a *HindIII* deletion was carried out to give the vectors p1NIL and p2NIL (Fig. 1a). The pGOAL vectors were derived from pBR322 by first deleting the 1.9 kb *EcoRV*–*PvuII* fragment, then the 450 bp *EcoRI* fragment from Tropist4, containing two *PacI* sites, was inserted. Other marker genes were then introduced between the *PacI* sites. The *P_{hsp60}*–*sacB* gene was derived from p6.11 (Selwyn Quan, Glaxo Wellcome Research and Development, Stevenage, UK), *P_{Ag85}*–*lacZ* from pEM37 (Edith Machowski, South African Institute for Medical Research, Johannesburg, South Africa), hygromycin resistance gene (*hyg*) from pAGAN40 (Hinds *et al.*, 1999) and *lacZ* from pATB10 (Martin Everett, Glaxo Wellcome Research and Development, Stevenage, UK) (Fig. 1b and Table 2).

Construction of delivery vectors. The cloning strategy for generating suicide delivery vectors is illustrated in Fig. 2. For the *tlyA* deletion, a 2.5 kb *SmaI* fragment from pCIG19 (Wren *et al.*, 1998) was cloned into the *PmlI* site of p2NIL and a subsequent 207 bp *PmlI*–*EcoRV* deletion was made (Fig. 3a). The *PacI* cassette from pGOAL15 (*hyg*, *lacZ*, *P_{hsp60}*–*sacB*) was then cloned into the single *PacI* site to generate the suicide delivery vector pTLY5. For the unmarked *plcABC* deletion, two separate fragments were cloned into p2NIL. First a 2.0 kb *PstI* fragment (Fig. 3b) was cloned into the *PstI* site of p2NIL followed by a 2.0 kb *SmaI* fragment (Fig. 3b) in the *PmlI* site. This construct has the two fragments separated by a small region of the cloning vector. The marked deletion was constructed by cloning the *hyg* gene into the *Scal* site in the middle of the two fragments (pPLC8). The final delivery vectors were generated by adding the *PacI* cassette from

Fig. 2. Cloning strategy for generating suicide delivery vectors. (a) The target gene is cloned into one of the pNIL vectors and the required mutation generated. (b) The *PacI* cassette containing the desired marker genes is then excised from the appropriate pGOAL vector and cloned into the unique *PacI* site of the pNIL/mutated gene vector, resulting in the final suicide delivery vector. (c) The final vector thus contains *oriE*, the kanamycin resistance gene (*kan*) and the *f1* origin (*f1 ori*).

pGOAL15 (*hyg* *lacZ* *P_{hsp60}*–*sacB*) to the *plcABCΔ* construct to give pPLC9, the *PacI* cassette from pGOAL13 (*lacZ* *P_{hsp60}*–*sacB*) to the *plcABCΔ::hyg* construct to give pPLC10 and the *PacI* cassette from pGOAL19 (*hyg*, *P_{Ag85}*–*lacZ* *P_{hsp60}*–*sacB*) to the *plcABCΔ* construct to give pPLC11 (Fig. 2b, c).

Isolation of mutants. Vector DNAs were pretreated with 100 mJ UV light cm^{-2} (Hinds *et al.*, 1999) and used to electroporate *M. tuberculosis* (Parish *et al.*, 1999). *hyg^R* *kan^R* transformants (single cross-overs) were streaked out onto plates containing either hygromycin (for marked mutations) or plates without selection (for unmarked mutations). A

Fig. 3. Delivery vectors used to generate *M. tuberculosis* mutants. (a) Unmarked *tlyA* mutation: a 207 bp *PmlI*-*EcoRV* deletion was made in the *tlyA* gene (black) and the *hyg*, *lacZ* and *P_{hsp60}-sacB* cassette from pGOAL17 was added to generate pTLY5. (b) *plcABC* deletion mutations. The *SmaI* and *PstI* flanking regions of the *plcABC* gene cluster were cloned into p2NIL. The *hyg*, *lacZ* and *P_{hsp60}-sacB* cassette was added to generate pPLC9 and the *hyg*, *P_{Ag85}-lacZ*, *P_{hsp60}-sacB* cassette was added to generate pPLC11. The marked mutation was made by cloning the *hyg* gene into the middle of the two flanking regions and the *lacZ*, *P_{hsp60}-sacB* cassette added to make the final delivery vector pPLC10. The region absent from the delivery vectors is shown in black. E, *EcoRV*; P, *PmlI*; Ps, *PstI*; S, *SmaI*.

loopful of cells was then resuspended in liquid medium by rigorous vortexing with 1 mm glass beads and serial dilutions plated onto sucrose plates (plus hygromycin and X-Gal where appropriate). Plates were incubated for 3–4 weeks. *suc^R* colonies were then streaked onto plates with and without kanamycin (plus hygromycin where appropriate) to identify *kan^S* colonies.

Genotypic analysis. DNA was prepared from *kan^S* colonies by the methods of Belisle or Santos (Belisle & Sonnenberg, 1998; Santos *et al.*, 1992). Both Southern blotting and PCR analyses were carried out to confirm the expected genotype. For the *tlyAΔ* mutant, genomic DNA was digested with *XhoI* and hybridized to a probe corresponding to the entire *tlyA* gene. Strains carrying the deletion lost the wild-type bands of 3.3 and 0.8 kb and gained a band of 3.9 kb, reflecting the loss of an *XhoI* site. In addition, PCR using primers which flanked the deletion site was carried out, producing a 448 bp PCR fragment in the wild-type and a 241 bp fragment in the deletion strains. Genomic DNA from the *plcABC* mutant strains was digested with *EcoRI* and hybridized to the *PstI* fragment shown in Fig. 3(b) (partial *plcA* gene and flanking region): the wild-type possessed a 3.2 kb band, the deletion strain had a 5.7 kb band and the marked mutation had a 4 kb band. PCR analysis was also carried out using several sets of primers: set one was designed to amplify a 0.7 kb fragment from the wild-type which was absent from both mutant strains, set two amplified a 1.4 kb fragment from the unmarked deletion strain only and set 3 amplified a 1.1 kb fragment from the marked deletion strain only.

RESULTS

A rapid cloning strategy

We have been developing an efficient system for introducing defined mutations in *M. tuberculosis*, but have

found that the construction of the suicide delivery vectors has been problematic. This is firstly due to the lack of suitable restriction enzyme sites when multiple markers are already present, making gene cloning and manipulation difficult. Second, we often wish to change the marker genes used and this can entail a completely new cloning exercise. To overcome this bottleneck we have developed a flexible cloning strategy based on two different vector series to separate out the cloning and manipulation of the target gene from the inclusion of the required marker genes.

The first series of plasmids (pNIL) is for manipulating the gene of interest (Fig. 1a). The plasmids consist of a simple cloning vector with an origin of replication for *Escherichia coli* (*oriE*), a kanamycin resistance gene (*kan*) and multiple cloning sites (MCSs) with different restriction enzyme sites, but including a single *PacI* restriction enzyme site. The second series (pGOAL) is used to generate and store a number of cassettes of marker genes (Fig. 1b and Table 2). These consist of vectors containing *oriE*, an ampicillin resistance gene (*amp*) and different combinations of marker genes (*hyg*, *lacZ* and *sacB*) flanked by two *PacI* sites. Thus the marker genes can be excised as a *PacI* cassette and inserted into any of the pNIL series in a one-step cloning process. The benefit of using *PacI* sites is that none is present in the *M. tuberculosis* genome (Cole *et al.*, 1998) or in the marker genes used in this study.

Strategy for construction of delivery vectors

The general strategy for constructing suicide delivery vectors is shown in Fig. 2. One approach is for the gene of interest to be cloned into a pNIL vector, the choice depending on which restriction sites are suitable. The required mutation is then generated, either by insertion of an antibiotic resistance gene, such as *hyg*, or by constructing an unmarked mutation (e.g. pTLY5; Fig. 3a). Alternatively, two separate gene fragments and flanking DNA can be brought together, effectively resulting in a deletion (e.g. pPLC10; Fig. 3b). The other marker genes such as *lacZ*, *sacB* and *hyg* are then cloned in as a *PacI* cassette from the appropriate pGOAL vector (Table 2).

Construction of *M. tuberculosis plcABC::hyg, plcABCΔ* and *tlyAΔ* mutants

To test the vectors out, constructs were made carrying marked and unmarked *plcABC* deletions (pPLC10 and pPLC9, respectively) and an unmarked *tlyA* deletion (pTLY5) (Fig. 3). Mutants were then generated by a two-step process for both marked and unmarked mutants. Single cross-overs were initially obtained and the second cross-over event (to generate double cross-over mutants) was subsequently selected and/or screened for by using the *lacZ*, *sacB* and *kan* genes. Vectors were pretreated with UV light and electroporated into *M. tuberculosis* and transformants were selected on hygromycin/kanamycin/X-Gal plates. Ten transformants were isolated after electroporation with

pTLY5 and 50 each for pPLC9 and pPLC10. Unexpectedly, all the colonies were white, suggesting that the *lacZ* gene was not being expressed in *M. tuberculosis*. Southern blotting confirmed that representative colonies were single cross-overs.

The purpose of the *lacZ* gene had been to distinguish single cross-overs from double cross-overs in the second stage; in the absence of adequate expression, we used kanamycin sensitivity as a screen. One single cross-over transformant was picked from each plate and streaked out onto plates lacking antibiotics. Following growth, a loopful of cells was resuspended in liquid medium and plated onto sucrose plates to select for cells which had lost the integrated plasmid through a second cross-over. A reduction in c.f.u. of approximately 10^4 was seen on plates containing sucrose as compared to plates containing no sucrose. *suc^R* colonies were streaked onto plates with and without kanamycin and scored for growth after 2–3 weeks to distinguish double cross-overs from single cross-overs which had acquired spontaneous resistance to sucrose. DNA was prepared from *kan^S* colonies and analysed by Southern blotting and PCR.

For the marked *plcABC::hyg* mutation, colonies which have the phenotype *hyg^R kan^S suc^R* should all be double cross-over mutants; 12/45 *suc^R* colonies were *kan^S* – six of these were analysed by Southern blotting and PCR and all had the expected mutant double cross-over genotype.

For the unmarked mutations, a second cross-over can result in either the wild-type allele or the mutant allele remaining in the chromosome, depending on where the second event occurs. Thus, *kan^S hyg^S suc^R* colonies isolated can carry either the wild-type or mutant allele. The relative frequency of the two will be dependent on the relative frequency of recombination on each side of the gene and the relative fitness of the mutant and wild-type strains. For *tlyAA*, 12/48 *suc^R* colonies were *hyg^S kan^S* – 6/6 analysed by Southern blotting and PCR were all mutant double cross-overs. For *plcABCΔ* 20/40 *suc^R* colonies were *hyg^S kan^S* – 1/8 was the mutant double cross-over, with the remainder being wild-type.

Second generation pGOAL vectors

Since the *lacZ* gene was not expressed in *M. tuberculosis* in the existing pGOAL cassettes, we used the mycobacterial antigen 85A promoter to drive expression of this gene. We replaced the *lacZ* gene in pGOAL13 and pGOAL15 with the *P_{Ag85}-lacZ* gene fusion from pEM37 to create pGOAL17 and pGOAL19, respectively.

Construction of an unmarked *M. tuberculosis tlyAA plcABCΔ* mutant

One of the benefits of using unmarked mutations is that multiple mutations can be made in one strain by sequential mutagenesis using the same selection system. To show this to be a practical proposition, we used

the improved pGOAL vectors to introduce a *plcABC* mutation into the unmarked *tlyA* mutant described above.

The *PacI* cassette in pPLC9 was replaced by the *hyg*, *P_{Ag85}-lacZ*, *P_{hsp60}-sacB* cassette from pGOAL19 to make pPLC11. UV-pretreated DNA was used to transform the *M. tuberculosis tlyAA* mutant. The *hyg^R kan^R* single cross-over transformants obtained were blue on X-Gal plates, confirming that *lacZ* was being expressed from the *Ag85* promoter. A single cross-over colony was streaked out and a loopful of cells resuspended in medium and plated onto sucrose X-Gal plates. Approximately 10% of the *suc^R* colonies were blue (and were therefore spontaneous sucrose mutants) and 90% were white. These white colonies should all be double cross-overs and this was verified by patch testing them for *kan^S* – 40/40 were *kan^S*, confirming plasmid loss. *kan^S* colonies were analysed by Southern blotting and PCR and 4/18 were shown to be mutant double cross-overs. Thus, this mutagenesis strategy had successfully generated a double unmarked *tlyAA plcABCΔ* mutant.

DISCUSSION

An efficient, reliable system for making mutants

The system that we have used to generate mutants is designed to be highly efficient. The use of the two plasmid cloning systems allows the easy construction of the suicide delivery vectors. A two-step strategy means that only one single cross-over recombinant is required from the electroporation and the inclusion of multiple markers allows for the selection or screening for cells in which the second cross-over has occurred. This is particularly important where the mutant does not have a screenable phenotype. Recently, the generation of an unmarked mutation of *lysA* has been reported for *M. tuberculosis* (Pavelka & Jacobs, 1999). This strain was generated using a two-step strategy, with the double cross-over mutant strain being identified by virtue of its auxotrophy. However, this method is not applicable where the mutant has no obvious phenotype, whereas the use of multiple markers in the delivery vector enables screening for the second cross-over event.

We have now isolated mutant strains using a range of delivery vectors, all of which have been effective. The use of a system such as this, together with the efficiency and reliability of the technique should greatly facilitate the rapid generation of new mutant strains.

Flexibility of the two-step system

The cloning system described here is extremely flexible. Marker cassettes can be easily switched in the final delivery suicide vector if different combinations are required. In addition, new marker genes can be readily incorporated into the pGOAL *PacI* cassettes. Although these vectors were created specifically for mycobacteria, the general approach should be applicable to all bacterial species. The important features of the two-vector system

are the flanking of marker genes by a restriction site not found in the genome of the target organism, a simple MCS and the use of different antibiotic resistance markers in the two separate vector series.

Advantage of the two-step strategy

The use of a two-step strategy enables the isolation of unmarked mutants, which cannot be isolated using a one-step strategy. This has allowed us to make deletions of several genes, *plcABC* and *tlyA*, separately and in combination. A one-step strategy can be used to generate marked mutations, but in some cases the required mutants will not be obtained, for example if the gene is essential or the recombination frequency at that locus is low. In these cases a two-step strategy will be required, so it may be easier to start with this strategy, regardless. In addition, the two-step method for generating marked mutants is simpler, since all the double cross-overs isolated should have the mutant allele.

As we are using a two-step process only one single cross-over transformant is required. Thus, the use of larger vectors (containing several marker genes) which will reduce transformation efficiency does not pose a problem.

We have previously shown that the *lacZ* gene is a useful marker for distinguishing single cross-overs from spontaneous hygromycin resistance in *M. tuberculosis* (Parish *et al.*, 1999). Alternatively, using two antibiotics (kanamycin and hygromycin), as with the initial vectors (pTLY5, pPLC9 and pPLC10), abolished the problem of spontaneous antibiotic resistance. In either case, all the transformants that were recovered and analysed were single cross-overs.

The use of both *lacZ* and *sacB* together reduced the number of colonies to be tested for double cross-overs. When used individually there are problems with both of these markers. Although the *sacB* gene has previously been used for negative selection in mycobacteria (Pelicic *et al.*, 1996), problems have been reported with the high frequency of spontaneous sucrose resistance (Papa-vasanasundaram *et al.*, 1998; Pavelka & Jacobs, 1999; Pedulla & Hatfull, 1998). Our results show that 10–90% of *suc^R* colonies are not double cross-overs, so a second selection or screening marker is desirable. In our early experiments where *lacZ* was not expressed, we used the loss of the *kan* gene as a second quick screen. The later vectors with P_{Ag85}-*lacZ* added another way of immediately identifying those cells which were still single cross-overs. This minimizes the number of colonies to be analysed by Southern blotting and significantly reduces the overall workload. The expression of *lacZ* can be variable and some colonies will be pale blue, so again it cannot be relied on alone as an indication of double cross-overs. In addition, it cannot be used to select against single cross-overs and so needs to be used in conjunction with a negative selection marker. The *kan* gene provides a further way of rapidly screening out single cross-overs and any mixed single cross-over/double cross-over colonies.

Importance of unmarked mutations

The ability to generate unmarked mutations is of great importance in the drive towards generating new potential vaccine strains, where antibiotic resistance markers cannot be left in the chromosome. Unmarked mutations have several advantages. Secondary mutations can be made without the problem of finding another resistance marker, as we have shown by making a double mutant (*plcABCA tlyAΔ*). In addition, more subtle mutations can be made, such as point mutations to study gene function.

Mutant phenotypes

We previously described TlyA from *M. tuberculosis* (Wren *et al.*, 1998), which shows homology to a pore-forming haemolysin of *Serpulina hyodysenteriae*, which has been shown to play a role in virulence of the latter organism (Hyatt *et al.*, 1994). Three genes have been identified in the *M. tuberculosis* H37Rv genome which encode phospholipases C (Cole *et al.*, 1998) and haemolytic phospholipase C activity has been demonstrated for *M. tuberculosis* (Johansen *et al.*, 1996). Phospholipases C have been shown to be virulence factors for other pathogens (Songer, 1997; Titball, 1993). For example, in *Listeria monocytogenes*, PlcCs, together with a haemolysin (listeriolysin), are required for the escape of the organism from the phagosome and extracellular spread. In *Clostridium perfringens*, phospholipase C mutants show reduced virulence. *Pseudomonas aeruginosa* also possesses two phospholipases C which show homology to two of the three *M. tuberculosis plc* genes. We are currently investigating the virulence of the mutant strains in both animal and macrophage model systems.

Conclusions

We have developed a rapid cloning strategy to facilitate the construction of delivery vectors and have used these vectors to generate four mutant strains of *M. tuberculosis*, including a double unmarked mutant strain. The mutant strains generated in this study are now being analysed phenotypically in virulence studies.

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