Isolation of a novel insertion sequence from *Mycobacterium fortuitum* using a trap vector based on inactivation of a lacZ reporter gene

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An insertion sequence of *Mycobacterium fortuitum* has been isolated using a trap vector following insertion in and inactivation of the lacZ reporter gene. The trap vector is a temperature-sensitive (ts) *Escherichia coli*–mycobacterium shuttle plasmid, pCD4, which contains ts oriM, the kanamycin-resistance gene as a selection marker and a lacZ expression cassette. The ts mutation present in pCD4 functions in mycobacteria and enables screening for transposable elements from the mycobacterial genome that disrupt the lacZ gene by screening for white colonies on X-Gal plates in both mycobacterial as well as *E. coli* hosts. The vector was used to isolate a novel 1·653 kb insertion sequence from *M. fortuitum* named IS219. IS219 duplicated host DNA at the target site, had inverted repeats at its ends and contained two ORFs on one strand. One of the predicted proteins showed homology to a putative transposase from *Acetobacter pasteurianus*. IS219 was present in two copies in the genome of *M. fortuitum*. The trap vector appears to be useful in trapping insertion sequences from different mycobacteria by screening for the disrupted LacZ phenotype.

Keywords: insertion sequence, *Mycobacterium fortuitum*, reporter gene, molecular trap

INTRODUCTION

Insertion sequences (ISs) and transposons are emerging as excellent tools in genetics and pathogenesis studies of mycobacteria. They have been identified as markers for diagnosis and epidemiology studies, as the majority of them are species specific or are present over a narrow host range which make them useful as reliable strain-specific probes (Kunze et al., 1991; Collins et al., 1993; Small & Van Embden, 1994; Mazurek et al., 1991; McAdam et al., 1994; Mahillon & Chandler, 1998). Few IS-like sequences have been successfully exploited for insertional mutagenesis (Cirillo et al., 1991; Guilhot et al., 1994; Pelicic et al., 1997) and to integrate foreign genes into mycobacterial genomes (England et al., 1991; Dellagostin et al., 1993). The usefulness of these elements warrants the continued search for such elements from different mycobacterial species, with particular attention to pathogenic mycobacteria.

Most ISs in mycobacteria have been identified as IS-like elements found within repetitive DNA (McFadden et al., 1987; Thierry et al., 1990), by hybridization to drug-resistance markers (Martin et al., 1990) or through transposon traps (Cirillo et al., 1991; Guilhot et al., 1992). The second approach led to the isolation of transposon Tn610 from *Mycobacterium fortuitum* (Martin et al., 1990). Transposon trapping appears to be the most attractive alternative as it allows isolation of a true IS element through transposition into a reporter gene whose inactivation can be easily monitored. We have described in this paper an IS trap system that uses a temperature sensitive (ts) mycobacterium–*Escherichia coli* shuttle vector, pCD4, to trap ISs in the lacZ reporter system during replication in the mycobacterial host. Two clones with disrupted lacZ genes were isolated as...
white colonies on X-Gal plates in both mycobacterial as well as E. coli hosts. By using this vector, a new element, IS219, was isolated from M. fortuitum.

METHODS

Bacterial strains, plasmids and growth. M. smegmatis mc²155 (a high-frequency transforming strain) was a generous gift from Dr W. R. Jacobs, Jr, Albert Einstein College, New York, USA. Plasmid pMV261::lacZ (Stover et al., 1991) was kindly provided by Dr C. K. Stover (PathoGenesis Corporation, Seattle, USA). All transformations in E. coli were performed in strain DH5α (BRL). M. fortuitum was a clinical isolate. The construction of plasmid pCD4 is described below. E. coli strains were grown in Luria (LB) broth or on LB agar. Mycobacterial strains were grown in nutrient broth (NB), Middlebrook 7H9 (MB) or LB broth supplemented with glycerol (0.5 %) and on nutrient agar or MB agar. The media were supplemented with Tween 80 (0.05 %) to allow homogeneous growth of mycobacteria. When required, media were supplemented with either 25 µg kanamycin (Kan) ml⁻¹ or 80 µg X-Gal ml⁻¹. All media components were from Difco.

Genetic methods. For electroporation, mycobacterial cells were grown to an OD₆₅₀ of 1.0 in LB broth containing glycerol and Tween 80. The cells were electroporated as described by Connell (1994). Competent E. coli cells for transformation were prepared by the standard calcium chloride/rubidium chloride method (Sambrook et al., 1989).

Plasmid isolation was done by the alkaline-SDS lysis procedure (Sambrook et al., 1989). Plasmid DNA from mycobacterial cells was isolated by the alkaline-SDS lysis method with slight modifications. Cells were harvested at early stationary phase and inactivated at 80 °C for 1 h prior to isolation of plasmid DNA. Sequential incubation in lysozyme (1 mg ml⁻¹) and SDS/NaOH solution (10 min at 50 °C) and high salt/acetate mix (16–18 h) were done. Plasmid DNA was precipitated with 2-propanol.

For Southern blot hybridization, chromosomal DNA from mycobacterial strains was isolated as described by Connell (1994). The DNA was digested with restriction enzymes and electrophoresed through 0.8 % agarose in TAE (Sambrook et al., 1989). The fragments were transferred to a nitrocellulose membrane overnight by capillary transfer. The DNA was cross-linked to the membrane by using a UV Stratalinker (Stratagene). The DNA probe was labelled with digoxigenin; labelling and detection were done as described by Boehringer Mannheim. Restriction enzymes, biochemicals, T4 DNA ligase etc. were purchased from Promega, New England Biolabs and Sigma.

Cloning and subcloning of the IS for sequencing purposes was done using standard methodologies. DNA sequences were determined from double-stranded plasmid DNA on an automated DNA sequencer (ABI PRISM model 377; Applied Biosystems). Universal forward and reverse M13 and internal primers were used to sequence fragments cloned in pUC vectors. The sequences were analysed using the DNAseq package (Pharmacia). The BLASTX program (Altschul et al., 1997) was used for homology searches within the GenBank/EMBL databases.

Construction of pCD4. M. smegmatis mc²155[pMV261::lacZ] was exposed to 66 µg NTG ml⁻¹ for 45 min to give 5% survival (Holland & Ratledge, 1971). The cells were washed and plated on NA plates containing Tween 80 and Kan at a density of 300–400 c.f.u. per plate and incubated at 30 °C until the colonies were visible (3 d). The plates were then shifted to 40 °C, which prevented clones carrying plasmids with a ts mutation to grow in size. After confirmation of their ts nature in mycobacteria, the plasmids were transformed into E. coli. The stability of lacZ was checked with respect to the parent pMV261::lacZ plasmid by looking at the segregation of blue colonies into white and blue colonies in the presence of X-Gal after 72 h growth at 30 °C. One of the ts plasmids was named pCD4 and used for further studies.

Molecular trapping of ISs from M. fortuitum. The ts plasmid pCD4 was electroporated into M. fortuitum and blue colonies were selected on MB agar plates containing Kan and X-Gal at 30 °C. One such blue clone was grown in liquid medium at 30 °C in the presence of Kan for 72 h. Dilutions of the culture were plated to obtain well isolated colonies. Some white clones obtained were analysed for insertion in the lacZ expression cassette by examination of the BamH1 restriction profile. Further localization of the insert was performed using a combination of restriction enzymes.

RESULTS AND DISCUSSION

Effect of a ts mutation on lacZ expression in pCD4

The parent plasmid pMV261::lacZ showed a very unstable expression of lacZ when introduced into mycobacteria due to rapid random deletions in this expression cassette. Inactivation of the LacZ phenotype in pMV261::lacZ after 72 h growth at 37 °C was observed at high frequency (2 × 10⁻⁶), which largely resulted from deletions within or around the lacZ gene (Kumar et al., 1998). In comparison, the frequency of white clones was 5 × 10⁻⁵ for pCD4 as a result of a ts mutation. The frequency was identical at 30 and 37 °C. As the restriction profile of pCD4 was identical to the parent plasmid, many of the properties of the parent plasmid are expected to be retained. The ts character was observed only in mycobacteria. In E. coli, the cells were able to grow on Kan plates even at high temperature (40 °C), indicating that the ts mutation was present in the mycobacterial origin of replication. This was further confirmed by replacing the ts oriM with that of the parent plasmid, resulting in loss of the ts character in mycobacteria. The restriction profile of pCD4 is shown in Fig. 1.

Generation of DNA rearrangements in pMV261::lacZ. The M. smegmatis mc²155 host was monitored in the presence of a lower concentration of antibiotic (10 µg Kan ml⁻¹) at 30 °C to see if it could stabilize the plasmid in the population of cells. Growth at the lower concentration of Kan (10 µg ml⁻¹) did lower the frequency of white mutants (10–50-fold) but not to the extent observed with pCD4.

Isolation of ISs from M. fortuitum

M. fortuitum carrying pCD4 was grown in NB containing Kan and Tween for 72 h at 30 °C and plated on MB agar containing Kan and X-Gal. Both blue and white colonies appeared after 4–5 d. White colonies appeared at a frequency of 5 × 10⁻⁵. The plasmids from

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**Fig. 1.** Linear restriction map of pCD4, pCD48 and pCD49. The thick line represents \( \text{P}_{\text{hsp60}} \). The AccI–BamHI fragment of IS\(^{219} \) was used as a probe in Southern hybridization.

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**Fig. 2.** Agarose gel electrophoresis analysis of plasmids of 18 white mutants of *M. fortuitum* carrying pCD4 (lanes 2–19) and control pCD4 (lane 20). The plasmids were digested with BamHI and electrophoresed through a 1% agarose gel. BamHI digestion of pCD4 generates two fragments of 4-5 and 3-0 kb (marked by arrows). pCD48 (lane 5) and pCD49 (lane 14) show a shift in the size of the 4.5 kb fragment. HindIII restriction fragments of \( \lambda \) DNA (BRL) were run as size markers (lane 1).

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the white mutants thus obtained were analysed and their BamHI restriction profile inspected, using pCD4 as the control. BamHI cleaves pCD4 into two fragments of 3-078 (\( \text{lacZ} \)) and 4.5 kb (rest of the plasmid) (Fig. 2, lane 20). Plasmids from white colonies digested with BamHI yielded fragments of different sizes. Upon analysis they revealed either deletions within the \( \text{lacZ} \) structural gene or \( \text{P}_{\text{hsp60}}-\text{lacZ} \) region, deleting one BamHI site (Fig. 2, lanes 3, 4, 6, 8, 12, 13 and 19). In seven cases, the restriction profile remained the same (Fig. 2, lanes 2, 9, 10 and 15–18). When this gel was hybridized with *M. fortuitum* genomic DNA, only the upper bands in lanes 5 and 14 showed a signal. In these two cases, the 3-078 kb fragment remained the same but the 4.5 kb fragment was larger in size (Fig. 2, lanes 5 and 14) due to acquisition of DNA sequence from *M. fortuitum* as confirmed by Southern hybridization. These two plasmids were selected and named pCD48 and pCD49.

In *E. coli*, pCD48 and pCD49 were also Kan\(^r \) and formed white colonies in the presence of X-Gal. The BamHI profile of both plasmids remained the same whether they were propagated in *E. coli* or in *M. fortuitum*. Hence the IIs acquired from the genome during replication in *M. fortuitum* were stable and could be maintained in *E. coli*.

**Molecular analysis of pCD48 and pCD49**

The BamHI profiles of pCD48 and pCD49 indicated that the IIs were not located in the \( \text{lacZ} \) gene. Their location in the \( \text{P}_{\text{hsp60}} \) region was confirmed by digestion with BamHI, BglII, BamHI/BglII, HpaI and HpaI/XbaI (Fig. 1) and subsequent hybridization with genomic DNA from *M. fortuitum* (Fig. 3) and a \( \text{P}_{\text{hsp60}} \) fragment (not shown) as probes. The fragments in pCD48 and pCD49 representing junctions of the IS element and \( \text{P}_{\text{hsp60}} \) hybridized with both *M. fortuitum* genomic DNA and \( \text{P}_{\text{hsp60}} \) while true internal fragments hybridized only with *M. fortuitum*.

**pCD48.** In case of pCD48, the two 0-5 and 0-4 kb fragments obtained from BamHI/BglII digestion were
IS219 is 1653 bp long with 8 bp imperfect inverted repeats at its termini. Analysis of the nucleotide sequence revealed a G + C content of 63.7 mol%, which is typical of mycobacteria. Two major ORFs were identified, ORFA (bases 125–913) of 272 aa having a potential RBS 9 nt upstream of the start codon (GTG), and ORFB (bases 756–1656) of 300 aa with ATG as the start codon. A potential RBS is present 10 nt upstream of the start codon. Two direct repeats and two inverted repeats were found within IS219. Using the BLASTX program (Altschul et al., 1997), the putative mycobacterial protein encoded by ORFB showed 27% identity and 40% positive matches over 158 aa to a putative transposase from Acetobacter pasteurianus (Takemura et al., 1991) (Fig. 4) but no significant homology to any other known IS element was found. This suggests that the element discovered by us through the \textit{lacZ} trap system is a unique one not reported so far.

**IS219 is unique to \textit{M. fortuitum}**

Southern analysis of \textit{E. coli} K-12, \textit{Bacillus subtilis} and 14 strains of mycobacteria was performed by using the internal \textit{Bam}HI–\textit{Acc}l fragment of IS219 as a probe (Fig. 5). IS219 contains one \textit{Bam}HI site and one \textit{Acc}l site. Chromosomal DNA from different strains were digested with \textit{Bam}HI. Since the probe used is present in only one arm of IS219, each band obtained upon hybridization should indicate a single copy of IS219 in the genome. The hybridization was positive with \textit{M. fortuitum} DNA, revealing two bands, whereas no hybridization signals were obtained with \textit{Mycobacterium avium}, \textit{Mycobacterium chelonae}, \textit{Mycobacterium xenopi}, \textit{Mycobacterium smegmatis}, \textit{Mycobacterium simiae}, \textit{Mycobacterium szulgai}, \textit{Mycobacterium tuberculosis} H37Ra, \textit{M. tuberculosis} H37Rv, \textit{Mycobacterium asiaticum}, \textit{Mycobacterium scrofulaceum}, \textit{Mycobacterium bovis} BCG, \textit{Mycobacterium kansasi}, \textit{Mycobacterium aurum}, \textit{Mycobacterium phlei} (Fig. 5), \textit{E. coli} K-12 and \textit{B. subtilis}. The results were confirmed by PCR amplification (data not shown). The element appears to be present in two copies in \textit{M. fortuitum} and shows no homology to any known IS elements reported so far, including IS6100 sequences of \textit{Tn610} transposon reported from \textit{M. fortuitum} (Martin et al., 1990). Southern blot analysis with a probe internal to IS219 (Fig. 1) did not reveal the presence of a similar sequence in other mycobacterial species, including \textit{M. tuberculosis}, as also confirmed by \textit{M. tuberculosis} genome analysis. This indicates that the sequence is specific for \textit{M. fortuitum}.

**Concluding remarks**

In this investigation, a new IS element from \textit{M. fortuitum} has been isolated using an IS/transposon trap vector, pCD4. Two other trap systems in mycobacteria have been described. Cirillo et al. (1991) described isolation of IS1096 from \textit{M. smegmatis} inserted in a \textit{lacZ} reporter
Novel *M. fortuitum* insertion sequence

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*Fig. 4.* Alignment of potential translation product of IS219 ORFB with transposase (Tnpase) from IS1380 of *Acetobacter pasteurianus* (Takemura et al., 1991). + symbols in the consensus line indicate conservative substitutions.

*Fig. 5.* Southern blot hybridization of *Bam*HI-digested genomic DNA from different mycobacterial species with an internal *Bam*HI–*Acc*I fragment of IS219 which should hybridize to only one arm of the insertion element. Only *M. fortuitum* hybridizes to the IS219 probe.

**Gene**, which was integrated into the chromosome of *M. smegmatis*. White colonies resulting from insertion of an element in the *lacZ* gene were isolated at a low frequency $(8 \times 10^{-5})$. Isolation of an IS required its cloning from genomic DNA into *E. coli*, which could pose problems related to instability of the element (Kumar et al., 1998). Another similar system was described by Guilhot et al. (1992), which led to the isolation of IS6120 from *M. smegmatis*. The authors used a shuttle plasmid carrying an apramycin-resistance gene controlled by *cI*, the lambda phage repressor gene. The plasmid was replicated in *M. smegmatis* and then transferred into *E. coli*. The strain resistant to apramycin corresponded to *cI* inactivation as a consequence of insertion of a mobile element IS6120 during replication in *M. smegmatis*. The *pCD4* vector described in this study has several merits. The use of a plasmid *lacZ* reporter system allows the screening of a large number of colonies for inactivation in a single step, and does not require additional cloning. Plasmid DNA from the mycobacterial host could be directly transformed into *E. coli* and white mutants could be selected and analysed for the presence of ISs. Since the nucleotide sequence of the whole plasmid is known, the location of the element is very easy to demonstrate precisely. The presence of *hsp60* promoter-driven *lacZ* and *oriM* in *pCD4* allows its use in different mycobacteria, including *M. tuberculosis*, for isolation of ISs that can be stably maintained in *E. coli*. In this investigation, the use of *pCD4* indeed led to the isolation of an IS. In two out of 40 white mutants derived from *pCD4* during 72 h growth at 30°C, *lacZ* inactivation due to insertion of a sequence was demonstrated. Nucleotide sequencing of one of the sequences designated IS219 confirmed the typical features of IS elements like inverted repeats at the termini flanked by direct repeats and ORFs.
M. fortuitum is associated with a wide variety of infections involving lungs, skin, bone, central nervous system, prosthetic heart valves and also in disseminated disease (Silcox et al., 1981; Woods & Washington, 1987; Wallace et al., 1991). It will be interesting to investigate the occurrence of IS219 in different M. fortuitum strains to establish its use in diagnostic and epidemiological studies.

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