Two-dimensional bacterial genome display: a method for the genomic analysis of mycobacteria

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Annually, *Mycobacterium tuberculosis* is the cause of approximately three million deaths worldwide. It would appear that currently available therapies for this disease are inadequate. The identification of genes involved in mycobacterial virulence will facilitate the design of new prophylactic and therapeutic interventions. A method for high-resolution comparison of bacterial genomes has been developed to facilitate the identification of genes possibly involved in the virulence of clinically relevant mycobacteria. This 'two-dimensional bacterial genome display' (2DBGD) method utilizes two-dimensional DNA electrophoresis to separate, on the basis of size and G+C content, genomic fragments generated with different restriction endonucleases. The use of this method to identify genomic differences between species, strains and, most importantly, isogenic mutants of mycobacteria is reported. That 2DBGD can be used to identify differences resulting from either insertional mutagenesis using a gentamicin-resistance gene or from a frameshift mutation is demonstrated.

Keywords: *Mycobacterium avium* complex, tuberculosis, strain differentiation, fingerprint, two-dimensional DNA electrophoresis

INTRODUCTION

A number of species of mycobacteria are pathogenic in man. The causative agent of tuberculosis, *Mycobacterium tuberculosis*, is the leading cause of death due to a single infectious species, killing an estimated three million people annually. Mycobacteria belonging to the *Mycobacterium avium* complex (MAC), i.e. *M. avium* and *Mycobacterium intracellulare*, can also establish pulmonary infections in humans and are on the increase mainly as a result of the HIV epidemic (Bermudez *et al.*, 2000; Havlir & Barnes, 1999). *M. tuberculosis* and members of the MAC complex are facultative intracellular pathogens that can survive and replicate within macrophages. It is well established that the virulence of separate strains or isolates of *M. tuberculosis* or MAC can vary (Steenken *et al.*, 1934; Schaefer *et al.*, 1970; Collins & Smith, 1969; Collins & Stokes, 1987; North & Izzo, 1993). However, little is known, at a genetic level, of the virulence mechanisms employed by mycobacteria to evade the host immune system. We propose the use of a novel approach that utilizes two-dimensional DNA electrophoresis (2DDE) technology to compare genomes from different bacterial isolates that could be applied to identify mycobacterial virulence genes.

It has become feasible to electrophoretically separate and display, in two dimensions, DNA fragments derived from genomic digests. Separation in the first dimension is by fragment size and in the second dimension separation is by mobility in denaturing gradients. Through the appropriate choice of restriction enzymes, changes as small as single base point mutations can be visualized in 2D gels. This technique has already been successfully used to display microsatellite polymorphisms in the human genome for use in genetic mapping and in studying genomic alterations in animal models and human cancers (Lam *et al.*, 1996; Hughes *et al.*, 1998; Marcinek *et al.*, 1997). In addition, 2DDE analysis

Abbreviations: 2DBGD, two-dimensional bacterial genome display; 2DDE, two-dimensional DNA electrophoresis; DGGE, denaturing gradient gel electrophoresis; MAC, *Mycobacterium avium* complex.

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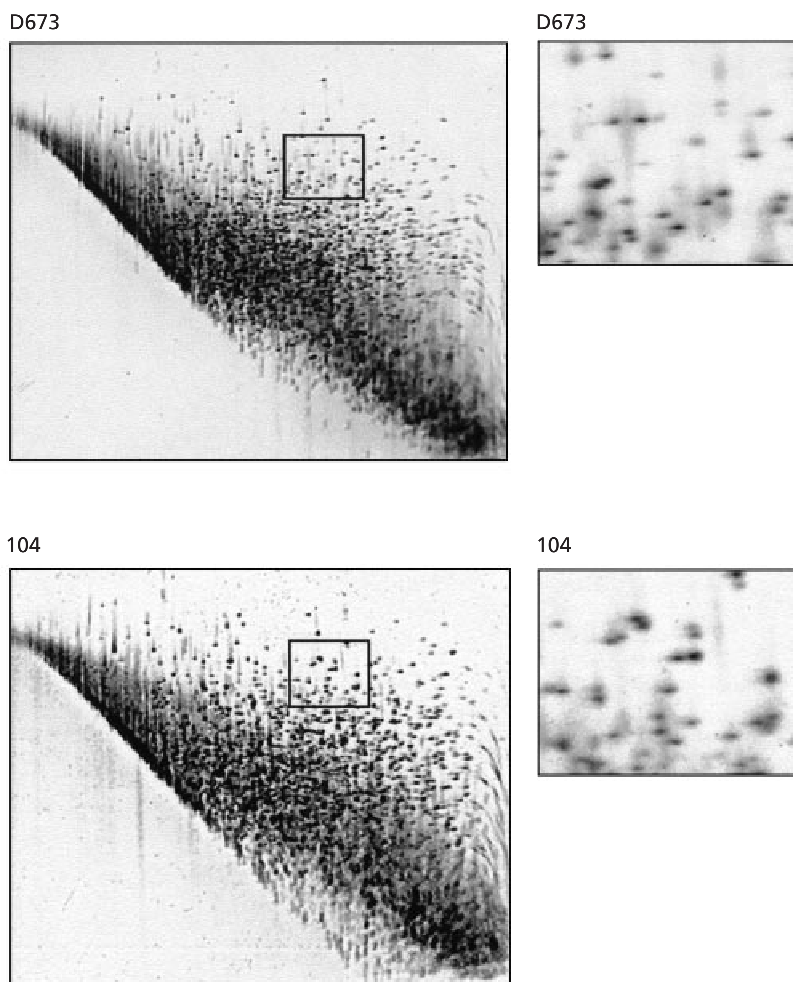

Fig. 1. 2DBGD displays of *Alu*I-digested genomic DNA from *M. intracellulare* D673 and *M. avium* 104 (large displays on the left). Regions of high spot density (the dark areas at the leading edge) can be resolved by altering electrophoresis conditions and using alternative restriction enzymes. Close-ups of a corresponding area from each 2DBGD display are shown for *M. intracellulare* D673 and *M. avium* 104. The two samples were run in parallel in separate denaturing gradient gels. The majority of spots do not align upon comparison of these two displays.

has been used to distinguish different strains of *Bordetella pertussis* (Malloff *et al.*, 2002).

We describe a method, two-dimensional bacterial genome display (2DBGD), for producing displays of mycobacterial genomes using 2DDE to separate genomic segments cut with various restriction endonucleases. We demonstrate the utility of this method by detecting genomic differences at the species and strain level and between isogenic mutants.

METHODS

Bacterial strains and DNA. *M. tuberculosis* H37Rv and H37Ra (Steenken *et al.*, 1934), *M. intracellulare* D673 (Dunbar *et al.*, 1968), D673-Katg (Marklund *et al.*, 1998), D673-19KDa (Mahenthiralingam *et al.*, 1998) and 1403 (ATCC 35761), and *M. avium* 104 (currently being sequenced by TIGR, www.tigr.org/tdb/) were grown in 7H9 broth (Difco) supplemented with oleic acid-albumin-glucose complex (OADC) plus 0·05 % Tween 80. OADC was prepared by dissolving 8·1 g NaCl, 50 g BSA and 20 g D-glucose in 950 ml dH_2O. The solution was then adjusted to pH 7·0 using NaOH. To this was added a 30 ml solution of 0·6 ml sodium oleate plus 0·6 ml 6 M NaOH in dH_2O. The OADC was warmed to 56 °C to clear and was filter-sterilized. Isolation of genomic DNA from mycobacteria was carried out using the method of Belisle & Sonnenburg (1998). Genomic DNA was digested with a variety of restriction enzymes that had been initially tested using the genome restriction digest tool of the Comprehensive Microbial Resource (Peterson *et al.*, 2001) to select ones that would produce an even distribution of fragments ranging from 200 to 2000 bp.

2D DNA electrophoresis. Five hundred nanograms of digested DNA fragments was treated with calf intestinal alkaline phosphatase (New England Biolabs) prior to radiolabelling with 35 kBq [γ-^{32}P]ATP (6000 Ci $mmol^{-1}$; Amersham Pharmacia Biotech) using T4 polynucleotide kinase (New England Biolabs). The resulting fragments were size-fractionated in 5 % non-denaturing acrylamide gels in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, 1 mM Na_2EDTA, 0·2 %, v/v, glacial acetic acid, pH 7·4) for 1600 volt-hours. Following this, each gel lane was cut and placed on top of a large format (25 × 20 cm) 6 % polyacrylamide denaturing gradient gel that contained an ascending gradient of formamide (10–40 %, v/v) and urea (1·8–7 M) in electrophoresis buffer. In the second dimension, parallel denaturing gradient gel electrophoresis (DGGE) was performed using an ISO-DALT apparatus (Amersham Pharmacia Biotech) for 1700 volt-hours and a constant temperature of 68·5 °C. DGGE gels were run in parallel in the same buffer chamber to ensure uniformity of electrophoretic conditions. A maximum of 10 gels can be run

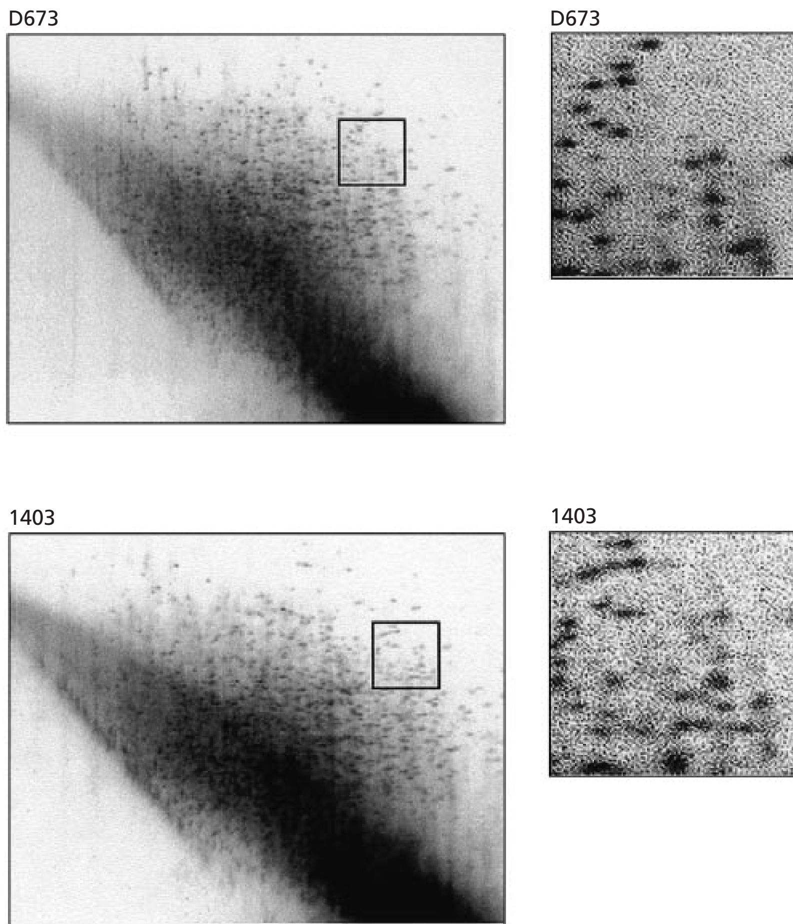

Fig. 2. Strain differentiation using 2DBGD. *M. intracellulare* D673 and 1403 were both digested with *Sau*3AI and run in parallel in separate denaturing gradient gels. A corresponding area (5 × 4 cm) from each gel (boxes in large display) shows that many of the spots do not align.

simultaneously in the ISO-DALT apparatus. The gels were dried prior to exposure to film. Alternatively, a gel could be left hydrated prior to electroblotting for Southern analysis.

2D gel comparisons were carried out by visual inspection. Spot constellations were easily aligned when comparing local areas of approximately 4 cm^2. Commercially available software for comparing 2D protein gels are suitable for such image analysis and comparison and we tested Malanie II (Bio-Rad) and NIH Image. However, in our experience, 2DBGD images were sufficiently reproducible that spot differences could be detected by simple visual inspection.

DNA probes and Southern hybridizations. Hybridizations were performed using positively charged nylon membranes (Roche). For Southern transfer of the DNA, 2D gels were electroblotted using a DALT blotting kit in the ISO-DALT electrophoresis tank. DNA probes for hybridization were generated using PCR amplification of DNA from *M. intracellulare* D673, *M. intracellulare* D673-19KDa and *M. intracellulare* D673-Katg. PCR amplification was performed under standard conditions with a programme of 30 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min and a final cycle of 72 °C for 10 min. Digoxigenin-labelled PCR products were generated for use as probes using oligonucleotides 5′-CACCTACCGCATCCACGAC-3′ $katG_{350\text{–}368}$, 5′-GGTCTCCTCGTCGTTCAT-3′ $katG_{806\text{–}788}$, 5′-GTTCGGGTGGTAACAAGTCG-$3'_{19Kdaf}$ and 5′-GCCGCTGATCTTGTAGCTGT-$3'_{19Kda\ rev}$. Prehybridization and hybridization was carried out according to the manufacturer's instructions (Roche).

RESULTS

We have used a wide range of mycobacterial species and strains in the development of this methodology. Our main purpose in developing the use of 2DBGD is for the identification of virulence determinants in mycobacteria. This technique enables a more subtle examination of the mycobacterial genome than other techniques currently available, which can be limited to detecting a gain or loss of DNA. In addition to detecting a gain or loss of DNA, 2DBGD can be used to identify small changes in DNA sequence as well as changes in intergenic regions.

Species differentiation

Various species of mycobacteria were resolved using 2DBGD. The conditions for resolution of each species were determined empirically. On each occasion, no two species of mycobacteria produced the same display, regardless of the enzymes used. The choice of enzymes for use in this work were selected using the *M. tuberculosis* H37Rv genome sequence (Cole *et al.*, 1998) and the genome restriction digest tool of the Comprehensive Microbial Resource (Peterson *et al.*, 2001) and included *Hin*fI, *Alu*I, *Sau*3AI, *Sau*96AI, *Afl*III and *Nco*I. Fig. 1 shows *Alu*I displays of the genomes of *M. intracellulare* D673 and *M. avium* 104. The result

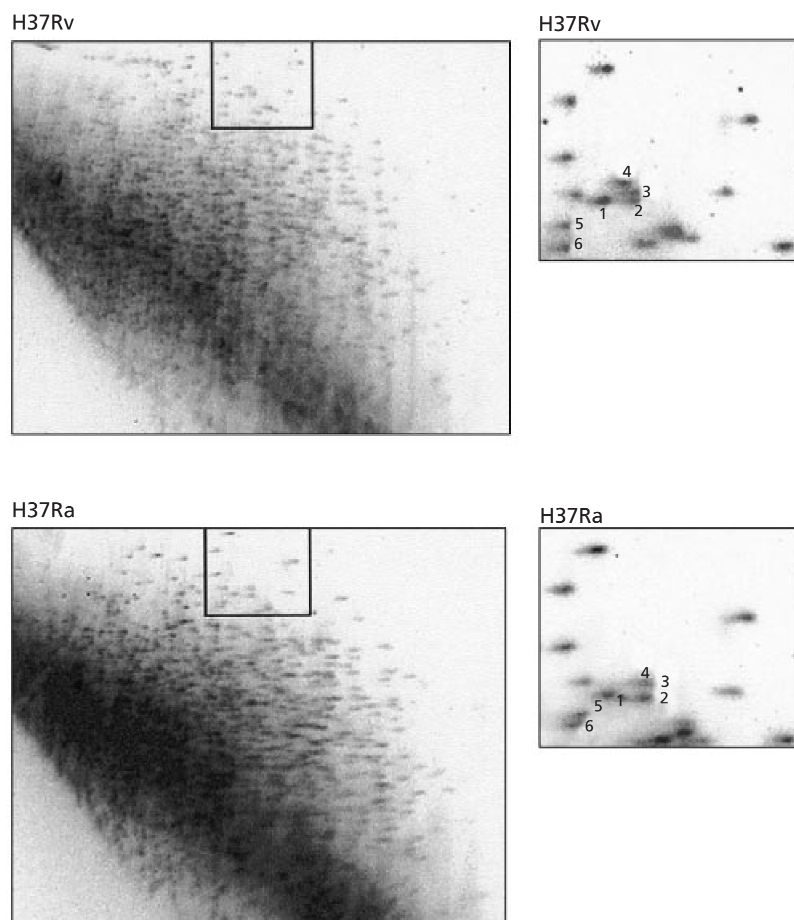

Fig. 3. Identification of differences in a virulent and avirulent laboratory strain of *M. tuberculosis*. *M. tuberculosis* H37Ra and H37Rv were subjected to 2DBGD after digestion with the restriction endonuclease *Hin*fI. The two samples were run in parallel in separate denaturing gradient gels. A corresponding area (2·5 × 3·5 cm) from H37Rv and H37Ra (boxes in large display) shows numerous spots that are identical between the two strains. However, one spot (4) that is present in H37Rv is absent from H37Ra while another spot (6) appears to migrate differently in the two strains. Other spots of interest are numbered 1, 2, 3 and 5.

obtained clearly demonstrates that the resolving power of this method can distinguish between different species of mycobacteria.

Strain differentiation

To investigate the ability of this technique to resolve strains of mycobacteria, two strains of *M. intracellulare*, D673 and 1403, were compared using 2DBGD. These strains originate from two independent clinical isolates obtained approximately 50 years ago. Interestingly, analysis of these strains by 2D-PAGE reveals fewer than 10 obvious differences over the whole proteome (L. A. Brooks, personal communication). However, Fig. 2 demonstrates that no area of *Sau*3AI displays of these two strains can be overlaid. Similar results were obtained with *Sau*96I displays (not shown).

The application of 2DBGD to compare closely related strains is demonstrated with *M. tuberculosis* H37Rv and its avirulent counterpart H37Ra, commonly used reference strains that are derived from the same parent strain, H37 (Steenken *et al.*, 1934). These strains are ideal candidates for the identification of virulence determinants. However, despite the many attempts to identify the genetic differences between H37Rv and H37Ra, which may explain their different virulence, the restoration of virulence to H37Ra using genes from H37Rv has not been demonstrated (Brosch *et al.*, 1999; Pascopella *et al.*, 1994; Rindi *et al.*, 1999, 2001; Schmidt *et al.*, 1998). The comparison of *Hin*fI-digested genomic DNA enabled the identification of several fragments (spots) of interest that highlighted differences between these two strains, of which one is shown in Fig. 3. These differences are currently being cloned and sequenced. Any novel virulence gene candidates will then be validated by site-directed mutagenesis.

Differentiation of isogenic mutants

To test the application of this method for detecting mutations, we subjected two isogenic mutants of *M. intracellulare* D673, which had been constructed in our laboratory, to 2DBGD.

The first mutant, D673-19KDa, was produced by insertional mutagenesis using a cassette containing the 19 kDa antigen (19Ag) gene of *M. intracellulare* D673 disrupted by the gentamicin-resistance (Gm^r) gene of pUC-GM (Mahenthiralingam *et al.*, 1998). The mycobacterial 19Ag is a highly expressed glycolipoprotein known to be immunodominant in infected patients and considered a candidate virulence factor (Young & Garbe, 1991). Southern analysis of *Afl*III-digested genomic DNA from *M. intracellulare* D673 and the 19Ag mutant was performed using a digoxigenin-labelled

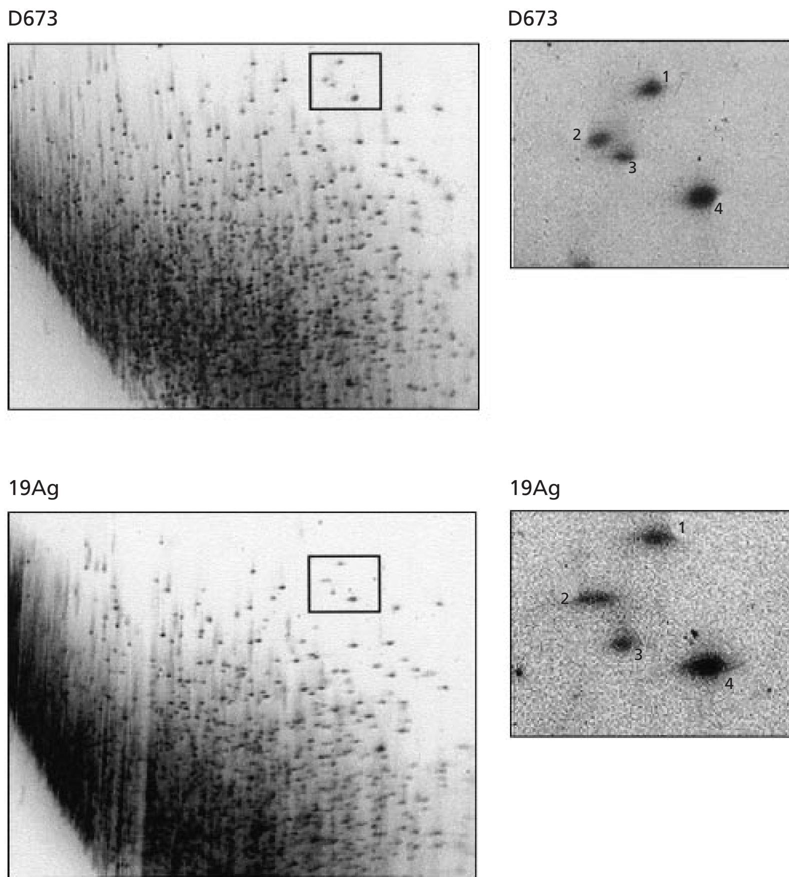

Fig. 4. Use of 2DBGD to identify a mutant produced by insertional mutagenesis using a gentamicin-resistance gene. Full displays of *M. intracellulare* D673 and the 19Ag mutant (19Ag) following digestion with *Alu*I are shown. Corresponding sections (2×2 cm) from 2DBGDs of D673 and the 19Ag mutant (boxes in large display) are shown for comparison. Spot 3 migrates differently in the two strains. Spots 1 and 4 are used for orientation purposes.

Gm^r cassette as probe. As expected, this produced two hybridization signals in the mutant but none in the parental D673 (data not shown). Therefore, this enzyme was a logical choice for resolution of this mutant, although digests using other restriction enzymes were also used to identify differences. Interestingly, regardless of which restriction enzyme was used with this mutant, between five and ten visible differences could always be identified, of which one is illustrated in Fig. 4.

The *katG* gene encodes an inducible, heat-labile catalase peroxidase suggested to protect mycobacteria from reactive oxidative metabolites produced by host phagocytes (Zhang *et al.*, 1992). Directed mutagenesis of the *katG* gene of *M. intracellulare* D673 was previously undertaken by generating a frameshift mutation at position 691 in the coding sequence, resulting in the loss of an *Nco*I site (Marklund *et al.*, 1998) to produce D673-Katg. Genomic DNA from this mutant was compared with *M. intracellulare* D673 using 2DBGD to test the ability of this method to identify very minor genomic differences. The fusion of the *Nco*I fragments at this position produced a 12·5 kb fragment, which is too large to be resolved by 2DBGD. However, comparison of *Alu*I displays of D673 and the KatG mutant revealed two shifted spots of approximately 450 bp (Fig. 5). Southern analysis confirmed the presence of the *katG* gene in the area of these spots. The KatG display was transferred to positively charged nylon membrane and hybridized with a digoxigenin-labelled *katG* probe. This enabled orientation of the mutated DNA with the hybridized probe. Cloning and sequencing of the DNA represented in the spots is needed to confirm the identity of the differences.

DISCUSSION

The genetic basis of mycobacterial pathogenesis is poorly understood due in part to the difficulties associated with working with the slow-growing pathogenic species. Only in recent years have general recombinant DNA techniques become applicable to mycobacterial systems. Although genome sequence information is available for *M. tuberculosis* and *Mycobacterium leprae*, it would be unrealistic to expect that all strains of pathogenic mycobacteria will be sequenced in the near future and therefore we need to develop methodologies that will allow us to compare the genomes of individual isolates. The establishment of 2DBGD, a high-resolution genomic fingerprinting technique, will add to the repertoire of tools for identifying genetic alterations associated with pathogenicity.

A number of factors determine the resolution limits of 2DBGD; however, 500–1000 spots can be typically resolved. Electrophoresis conditions (time, voltage and temperature), steepness of the denaturation gradient and acrylamide concentration can all be adjusted to best resolve any specific size range and G + C content. There are, however, areas of poor resolution present in the

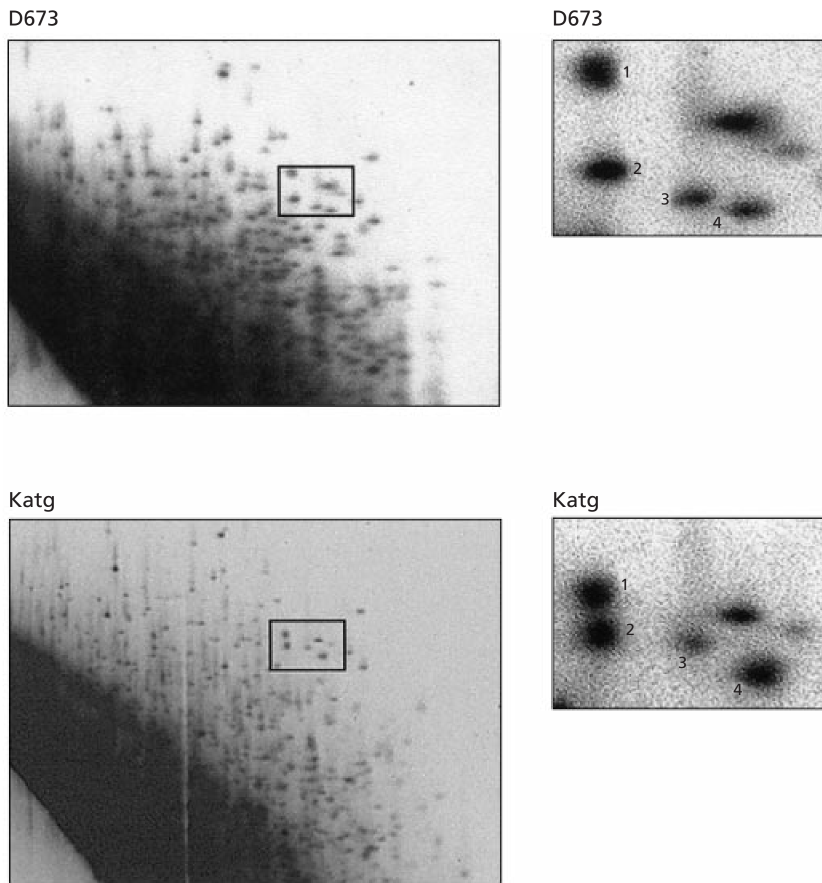

Fig. 5. Use of 2DBGD to identify a mutant produced by a frameshift mutation. Displays of *M. intracellulare* D673 and the KatG mutant (Katg) following digestion with *Alu*I are shown. A corresponding section ($2{\cdot}5\times 2{\cdot}5$ cm) from 2DBGDs of D673 and the isogenic mutant Katg is also shown (boxes in large display). Both spots 2 and 3 show different migratory patterns in the Katg mutant when compared to the parent D673. Hybridization with a probe specific for KatG located to the same area as spots 2 and 3. However, cloning and sequencing of the DNA represented in the spots would be needed to confirm definitively the identity of the spots. Spots 1 and 4 are used for orientation purposes.

displays, for example, at the leading edge of the gel where the density of fragments is too great for resolution. Theoretically, those fragments that are not well resolved under one set of conditions would be located in a different position of the display when an alternative enzyme or enzyme combination is used.

2DBGD is capable of comparing entire bacterial genomes and can identify large and small variations. The utility of 2DBGD, however, in comparing distantly related strains is limited because of the numerous small deletions or point mutations that are unrelated to the phenotype but result in significant differences in genomic displays (Figs 1 and 2). As an alternative, a new method called bacterial comparative genomic hybridization (BCGH) may facilitate the differentiation of such numerous inconsequential genetic alterations by resolving the reference and the test samples together on the same 2D gel. Following 2DBGD, the gel is then transferred to a positively charged nylon membrane, which is then hybridized sequentially with probes generated from the two samples. This technique produces two superimposable images generated from the same blot, which, when colour coded differentially, reveal the signals that are unique to either one or the other sample (Malloff *et al.*, 2001).

The utility of 2DBGD lies, mainly, in the identification of variations between the genomes of closely related strains, such as reported in this study for H37Rv versus H37Ra (Fig. 3) and for D673 and its mutants, D673-19KDa and D673-Katg (Figs 4 and 5). We deliberately selected two mutants constructed in our laboratory to enable us to check the power of 2DBGD to detect minor genetic differences. In addition, the hybridization with probes to the known mutation validated the visual identification in that they confirmed that the spots that differed between wild-type and mutant were fragments of the mutated gene.

Other genome comparison techniques such as microarrays, PFGE and genomic subtractive hybridization are powerful molecular tools. However, these methods are not designed to detect small deletions and insertions, point mutations, or genetic rearrangements. 2DBGD is sensitive enough for detecting such alterations and, unlike array technology, can identify the gain or loss of DNA from both test and reference strains. Furthermore, 2DBGD can identify minor changes in DNA sequence that would not be resolvable with techniques such as DNA microarrays or subtractive hybridization.

We have shown that the high GC genomes of mycobacteria can be resolved by 2DDE and demonstrated the utility of the 2DBGD technology in detecting genetic differences between species, strains and isogenic mutants of mycobacteria. Whilst we recognize that multiple

techniques will be required to fully unravel the genetic differences between virulent and avirulent forms of mycobacteria, 2DBGD represents a novel approach to this problem.

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