First Description of Natural and Experimental Conjugation between Mycobacteria Mediated by a Linear Plasmid

Michelle Christiane da Silva Rabello¹, Cristianne Kayoko Matsumoto¹, Luiz Gonzaga Paula de Almeida², Maria Carmen Menendez³, Rosangela Siqueira de Oliveira⁴, Rosa Maria Silva¹, Maria Jesus Garcia³, Sylvia Cardoso Leão¹*

¹ Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil, ²Laboratório Nacional de Computac¸a˜o Cientı´fica, Petrópolis, Brazil, ³Departamento de Medicina Preventiva, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain, ⁴Núcleo de Tuberculose e Micobacterioses, Instituto Adolfo Lutz, São Paulo, Brazil

Abstract

Background: In a previous study, we detected the presence of a Mycobacterium avium species-specific insertion sequence, IS1245, in Mycobacterium kansasii. Both species were isolated from a mixed M. avium-M. kansasii bone marrow culture from an HIV-positive patient. The transfer mechanism of this insertion sequence to M. kansasii was investigated here.

Methodology/Principal Findings: A linear plasmid (pMA100) was identified in all colonies isolated from the M. avium-M. kansasii mixed culture carrying the IS1245 element. The linearity of pMA100 was confirmed. Other analyses suggested that pMA100 contained a covalently bound protein in the terminal regions, a characteristic of inverton linear replicons. Partial sequencing of pMA100 showed that it bears one intact copy of IS1245 inserted in a region rich in transposase-related sequences. These types of sequences have been described in other linear mycobacterial plasmids. Mating experiments were performed to confirm that pMA100 could be transferred in vitro from M. avium to M. kansasii. pMA100 was transferred by in vitro conjugation not only to the M. kansasii strain from the mixed culture, but also to two other unrelated M. kansasii clinical isolates, as well as to Mycobacterium bovis BCG Moreau.

Conclusions/Significance: Horizontal gene transfer (HGT) is one of the most important mechanisms leading to the evolution and diversity of bacteria. This work provides evidence for the first time on the natural occurrence of HGT between different species of mycobacteria. Gene transfer, mediated by a novel conjugative plasmid, was detected and experimentally reproduced.

Introduction

Insertion sequences (ISs) are mobile genetic elements, capable of transposing and inserting at multiple sites in target DNA molecules [1]. The genus Mycobacterium contains a large number of different insertion elements, several of them found in members of the Mycobacterium avium complex (MAC) [2]. MAC organisms are ubiquitous in nature, and besides the classical species M. avium and Mycobacterium intracellulare, two new species were recently described: Mycobacterium chimaera [3] and Mycobacterium colombense [4]. M. avium is responsible for opportunistic infections in animals and humans, and has been frequently associated with disseminated infections in HIV-positive patients [5]. The insertion sequence IS1245 is a mobile element highly prevalent in subspecies of M. avium: M. avium subsp. avium, M. avium subsp. hominisuis, and M. avium subsp. silvaticum. Multiple IS1245 copies are usually present in M. avium clinical isolates, making this repetitive sequence a useful tool for epidemiological studies [6,7,8,9,10]. However, some studies have shown that this insertion element is sporadically present in other mycobacterial species, suggesting that it may be dispersed by horizontal gene transfer (HGT) [11].

Recently, our group confirmed the presence of the IS1245 element in colonies of Mycobacterium kansasii isolated from a M. avium-M. kansasii mixed culture from a bone marrow specimen of an HIV-positive patient [12]. Fifteen colonies were isolated from the mixed culture, making this repetitive sequence a useful tool for epidemiological studies [6,7,8,9,10]. However, some studies have shown that this insertion element is sporadically present in other mycobacterial species, suggesting that it may be dispersed by horizontal gene transfer (HGT) [11].

Recently, our group confirmed the presence of the IS1245 element in colonies of Mycobacterium kansasii isolated from a M. avium-M. kansasii mixed culture from a bone marrow specimen of an HIV-positive patient [12]. Fifteen colonies were isolated from the mixed culture, making this repetitive sequence a useful tool for epidemiological studies [6,7,8,9,10]. However, some studies have shown that this insertion element is sporadically present in other mycobacterial species, suggesting that it may be dispersed by horizontal gene transfer (HGT) [11].
these \textit{M. kansasii} colonies was confirmed by IS1245 amplicon sequencing and by detection of a 4,750-bp hybridization band in restriction fragment length polymorphism analysis using IS1245 as the probe (RFLP-IS1245) [12]. The results obtained strongly suggested that \textit{M. kansasii} acquired the IS1245 element from the \textit{M. avium} strain following an HGT event.

Conjugation, transduction and transformation are forms of HGT that occur naturally between bacteria. These three mechanisms have already been described or suggested in studies with mycobacteria, but information and experimental evidence are scarce. The majority of studies suggesting HGT in mycobacteria are based on comparative genome analyses [13]. Phages and plasmids were demonstrated to be the main vehicles of HGT and could contribute to IS dispersion among genomes. Many plasmids and bacteriophages have been described in the genus \textit{Mycobacterium} [14,15,16] and some mycobacterial plasmids were previously shown to carry IS elements [15,16]. Plasmid pCLP from \textit{Mycobacterium celatum} contains sequences similar to resolvases and transposases found in the \textit{Mycobacterium tuberculosis} genome [15]. Plasmid pMUM001 from \textit{Mycobacterium ulemanus} carries 26 copies of various ISs and IS fragments, 12 of which are known elements present in the chromosome of \textit{M. ulemanus} (4 copies of IS2404 and 8 copies of IS2606) [16]. Thus, IS jumping between bacterial chromosome and plasmids does not appear to be a rare event.

In this study, we investigated the mechanism of IS1245 element transfer from \textit{M. avium} to \textit{M. kansasii}, resulting in the detection of a 100-kb linear conjugative plasmid (pMA100).

\section*{Results}

\subsection*{Identification and characterization of a novel linear plasmid (pMA100)}

An extrachromosomal band of approximately 100 kb, designated pMA100, was identified by pulsed-field gel electrophoresis (PFGE) of undigested DNA with four \textit{M. avium} colonies and eight of the 11 \textit{M. kansasii} colonies from the original \textit{M. avium-M. kansasii} mixed culture, all of which produced amplicons by PCR-IS1245 (Figure 1A). The presence of IS1245 in pMA100 was confirmed by Southern blot hybridization of pulsed-field gels using a radioactivity-labeled IS1245 complementary probe (Figure 1B). Neither the 100 kb band nor any hybridization signal with the IS1245-derived probe was detected in the three \textit{M. kansasii} colonies that did not generate IS1245 amplicons (Figure 1A and 1B). A second hybridization band, undetected in PFGE gels stained with ethidium bromide, was observed with two of the eight PCR-IS1245 positive \textit{M. kansasii} colonies (88.14 and 88.15). This experiment was repeated and the results were reproduced (Figure 1B).

pMA100 co-migrated with linear lambda concatemers in PFGE using three different switch times, suggesting that it was a linear molecule (Figure 2A). Experiments with exonuclease III, exonuclease lambda and topoisomerase I confirmed the linearity of this molecule. pMA100 was sensitive to exonuclease III (which degrades DNA from free 3’ ends), and its migration was not affected by topoisomerase I, an enzyme that relaxes circular plasmids. Moreover, it was resistant to the action of exonuclease lambda, which degrades DNA from free 5’ ends, suggesting that pMA100 may have covalently bound proteins at its terminals (Figure 2B and 2C).

In plugs prepared without the addition of proteinase K to the lysis buffer, pMA100 did not enter the gel preparation during PFGE, suggesting again the presence of covalently bound proteins at its terminals (Figure 2D). Moreover, pMA100 was able to penetrate the gel after addition of 0.2% SDS, which denatures terminal end proteins (Figure 2E).

\subsection*{Partial sequencing of pMA100}

DNA from \textit{M. kansasii} (colony 88.8) digested with KpnI and analyzed by RFLP-IS1245 generated two hybridization fragments of approximately 3,500 bp and 4,100 bp, respectively (data not shown). Both fragments were cloned and sequenced. The analysis of the sequences obtained confirmed the presence of an intact copy of the IS1245 element in pMA100. IS1245 has a KpnI restriction site at position 499, and as a consequence, the first 499 bp of IS1245 were located in the ~4,100 bp KpnI fragment and 914 bp in the ~3,500 bp KpnI fragment. The IS1245 flanking region had >80% similarity to transposase-related genes present in other mycobacterial linear plasmids, namely the IS605 \textit{orfB} related sequence present in plasmid pMFLV01 from \textit{Mycobacterium gilvum} [17] and \textit{M. avium} (GenBank accession numbers NC_009339 and CP000065) and sequences related to \textit{Rv0922} and \textit{Rv0921} (IS1353) from \textit{M. tuberculosis} present in plasmid pCLP from \textit{M. celatum} [15]. The adjacent DNA sequences did not show similarity at the nucleotide level with other sequences in the GenBank database; however, it was possible to detect similarities at the amino acid level with two putative proteins described in the \textit{M. kansasii} genome (Figure 3).
Different mating experiments were carried out using M. avium colony 88.3, bearing pMA100, as donor and different recipient strains: M. kansasii colony 88.6, M. kansasii IAL 413, M. kansasii IEC 6805, M. bovis BCG Moreau or Mycobacterium smegmatis mc²155. Table 1 shows the results of PCR-IS1245 screening of 1,770 recipient strains colonies isolated after the mating experiments. Eight M. kansasii and 11 M. bovis BCG transconjugant colonies were detected by PCR-IS1245. No transconjugants were obtained when M. smegmatis mc²155 was used as recipient strain.

Additional experiments were carried out to confirm the presence of IS1245 and pMA100 in the transconjugant colonies. In each case, the presence of the 100 kb plasmid band, which hybridized with IS1245 was confirmed by PFGE (Figure 4A and 4B). Moreover, PFGE-DraI typing corroborated that each corresponding pair of recipient and transconjugant colonies belonged to the same strain (Figure 4C). Hybridization of DraI digested DNA from transconjugant colonies with the pMA100 derived probe was used to demonstrate that, besides the IS1245 element, the pMA100 molecule was also transferred to M. kansasii and M. bovis BCG in the mating experiments and that pMA100 did not integrate into the recipient strains chromosome (Figure 4D).

No transconjugants were detected in mating experiments performed using the rapidly-growing mycobacterium M. smegmatis mc²155 as recipient. A total of 1,200 colonies from three mating experiments, identified as M. smegmatis for the ability to grow on LB agar in less than 7 days, were screened by PCR-IS1245 but none produced IS1245 amplicons (Table 1).

Discussion

PFGE has been previously used for the detection of mycobacterial plasmids [17,18,19]. In the present study, PFGE allowed the identification of a 100-kb extrachromosomal band (pMA100) in PCR-IS1245 positive M. avium and M. kansasii colonies from a mixed culture, suggesting that pMA100 could be a plasmid responsible for the natural transfer of the IS1245 element between M. avium and M. kansasii.

This plasmid was shown to contain an intact copy of the IS1245 element and was able to be transferred between M. avium and other slow-growing species, such as M. kansasii and M. bovis BCG. The weak intensity of bands corresponding to pMA100 in PFGE gels suggests that it may be a low-copy number plasmid.

A second smaller IS1245-hybridizing band was observed by Southern blot hybridization with two PCR-IS1245 positive M. kansasii colonies (Figure 1B). It could be the same plasmid that underwent duplication and partial deletion. Otherwise, the IS1245 element could have transposed to a second plasmid present in M. kansasii. This supplementary copy was not submitted to further investigation. The transposition of this IS during in vitro culture passages was previously described in M. avium [20], and in M. kansasii [12]. As PFGE requires recent cultures, it is possible that further transposition events occurred in some of these colonies when they were cultivated again. IS1245 copies could have inserted in the chromosome.

The observation that pMA100 co-migrated with linear molecules in PFGE, and the results obtained with exonucleases and topoisomerase I support the notion that the 100 kb molecule is linear and suggest an invertin-like structure. Besides, our results suggest that pMA100 could contain a protein covalently bound to the 5’ ends. This is a characteristic of linear elements with inverted repeats at their ends such as the linear plasmid pCLP from M. celatum [19], also found in some actinomycetes, viruses, bacteriophages [21]. These elements belong to a class of genetic elements called invertrons [21]. Conjugative linear plasmids have been identified in Streptomyces spp. [22,23]. Because of this ability to conjugate, these elements can be spread among other actinomycetes, including mycobacteria and related organisms.

Partial sequencing of pMA100 confirmed that this plasmid contains an intact copy of IS1245, which is inserted in a transposase-related sequence homolog to orfB of IS605 from M. gilvum plasmid pMFLV01 (acc. number: CP000657) and to a transposase identified in M. celatum plasmid pCLP (acc. number: AF312688). IS605 is an insertion sequence reported frequently in strains of Helicobacter pylori, but has also been described in other bacteria [24]. The insertion of an IS within another IS is not a rare event, it has been described in M. tuberculosis [25] and other bacteria, such as Pseudomonas syringae [26], Rhizobium meliloti [27] and Escherichia coli K-12 [28]. It has been previously shown that the genomic insertion loci of IS1245 and IS1311, in M. avium strains carrying a single copy of any of these insertion sequences, are located in areas containing putatively truncated integrases and/or transposases [29]. Likewise, the transposase-related sequence

Figure 2. PFGE of DNA genomic preparations. (A) PFGE with undigested DNAs from M. avium 88.3 (1) and M. kansasii 88.8 (2) under different switch times, indicated below each figure; (B) pMA100 extracted from PFGE gels and treated with exconuclease III (3) or topoisomerase I; (C) pMA100 extracted from PFGE gels and treated (+) or not (-) with proteinase K to the lysis buffer; (D) DNA prepared with (+) or without (-) adding proteinase K to the lysis buffer; (E) same as in (D) in PFGE gels and running buffer prepared with 0.2% SDS. λ: DNA concatemers of the bacteriophage λ genome. doi:10.1371/journal.pone.0029884.g002

pMA100 was transmissible by conjugation to M. kansasii and Mycobacterium bovis BCG

Conjugation between Mycobacteria

PLoS ONE | www.plosone.org 3 January 2012 | Volume 7 | Issue 1 | e29884

Conjugation between Mycobacteria
identified in pMA100 could be a preferential locus for insertion of IS1245.

There are few studies and experimental evidence of HGT in the genus Mycobacterium. Reports on natural HGT in mycobacteria have been mostly based on circumstantial evidence, such as sequence similarities in phylogenetically distant organisms [15,30,31,32]. Recent genetic and genomic studies have provided evidence of the contribution of HGT to the emergence of members of the M. tuberculosis complex and Mycobacterium ulcerans [33,34,35]. M. ulcerans is phylogenetically related to Mycobacterium marinum and is thought to have diverged from a common progenitor by acquisition of the virulence plasmid pMUM001 [35]. Genome sequencing of the rapidly-growing mycobacterium Mycobacterium abscessus CIP 104536T, revealed that this strain acquired a full-length prophage containing non-mycobacterial genes and also a mercury resistance circular plasmid (23 kb) almost identical to plasmid pMM23 from M. marinum [36], thus suggesting the occurrence of HGT among mycobacteria. Promiscuous mycobacterial plasmids with the ability to propagate among different members of MAC have been identified, and they could promote the acquisition of genetic material through HGT in nature [15,37,38].

Experimental studies have demonstrated HGT in mycobacteria in conjugation and transformation events artificially induced in the laboratory. Gormley and Davies obtained the conjugative transfer of a mobile plasmid from *Escherichia coli* to *M. smegmatis* [39]. Conjugation-mediated Hfr (high frequency recombination)-like transfer of chromosomal DNA was described in *M. smegmatis*, with a mechanism distinct from that observed in *E. coli* Hfr strains and not mediated by plasmids [40]. Bhatt et al. showed spontaneous

---

**Table 1. Conjugation experiments using *M. avium* 88.3 as the donor strain.**

| recipient strain | conjugation conditions | screened recipient colonies | PCR-IS 1245 positive colonies |
|------------------|------------------------|-----------------------------|-----------------------------|
|                  | incubation temp (°C)   | time (days)                 |                             |
| *M. kansasii* 88.6 | 30                     | 10                          | 126<sup>1</sup>              | 1                           |
|                  | 37                     | 10                          | 180<sup>1</sup>              | 3                           |
| *M. kansasii* IAL 413 | 37                 | 10                          | 75<sup>1</sup>               | 1                           |
| *M. kansasii* IEC 6805 | 37               | 10                          | 150<sup>1</sup>              | 2                           |
| *M. bovis* BCG Moreau | 37             | *                           | 39<sup>1</sup>               | 11                          |
| *M. smegmatis* mc2155 | 37           | **                          | 1200<sup>1</sup>             | 0                           |

<sup>1</sup>three experiments with 15, 20 and 25 days incubations, respectively.

<sup>2</sup>three experiments with 2, 4 and 10 days incubations, respectively.

*pigmented colonies after exposure to light.
*PCR-IS 1245 positive.
*grown on LB agar.

**Figure 3. Gene features of the IS1245 flanking regions in pMA100.** The similarities identified in the 7,525-bp sequenced fragment of pMA100 are indicated. Similarities at nucleotide levels were identified using BLASTn and the regions of similarity are depicted as hatched bars; identities at amino acid level were identified using BLASTx and each region is shown as a white arrow, indicating the direction of transcription. Black bar localizes the IS1245 element. KpnI restriction sites are indicated.

doi:10.1371/journal.pone.0029884.g003
transference of hybrids of *Streptomyces coelicolor* conjugative plasmid SCP2* and *Mycobacterium fortuitum* plasmid pAL5000 from *Streptomyces coelicolor* or *Streptomyces lividans* to *M. smegmatis* in plate crosses. This transfer was DNase I sensitive and thus involved release of DNA from *Streptomyces* [41]. All these previous experiments involved only rapidly-growing mycobacterial species. The mating experiments described here demonstrated the transfer of the linear plasmid pMA100 and the IS1245 element between slowly-growing mycobacteria. We concluded that the most probable mechanism of acquisition of IS1245 by *M. kansasii* and *M. bovis* BCG in mating experiments was conjugation. The possibility that this transference occurred by transformation or transduction was not completely ruled out but would be very unlikely.

Transformation would require competent bacteria. There are no reports of slowly-growing mycobacteria being naturally competent and we did not carry out any procedure to produce competent mycobacteria before the mating experiments. High-efficiency transformation has been a major limitation in the study of mycobacteria and the most successful method to transform mycobacteria is by electroporation, which was not used here. Spontaneous plasmid transformation was observed with rapidly-growing *M. smegmatis*, but was limited to small plasmids, which were taken up intact, while larger plasmids suffered deletions [41]. We showed here that pMA100 was not transferred to *M. smegmatis*. It seems highly improbable that a linear molecule of ~100 kbp that might contain proteins attached to its ends could be easily and repeatedly transferred by natural transformation.

Transfection would require the presence of lytic phages and we never detected lytic plaques in any of our plates (data not shown). Repeated phage transduction of a unique 100 kb plasmid to different recipient bacteria would be highly unlikely. Otherwise, if the 100 kb molecule corresponded to phage DNA, it would have to integrate in the chromosome (lysogenic cycle) in order to be stably maintained in the recipient strains, and therefore would not be detected as an extrachromosomal molecule in PFGE gels, as shown in Figure 4.

Moreover, we have demonstrated that pMA100 is a linear molecule with an invertron-like structure. Invertrons were shown to be responsible for conjugation transfer in *Rhodococcus* [42,43,44] and *Streptomyces* [45,46]. Similarly to *Mycobacterium*, both genera belong to the class Actinobacteria.

According to the number of transconjugants carrying IS1245 in the mating experiment, the transfer frequency of pMA100 appears to be high under laboratory conditions. It is noteworthy that no transconjugants were obtained in mating experiments performed with *M. smegmatis* as the recipient bacterium. The analysis of more than 1,000 *M. smegmatis* colonies in mating experiments, suggests that this plasmid is not transferable from slow-growing to rapidly-growing mycobacteria. Even though this conclusion needs further confirmation, it may indicate that conjugative mechanisms could differ between slow- and rapid-growing mycobacteria.

In conclusion, natural and experimental conjugation-mediated HGT between different species of slow-growing mycobacteria was...
demonstrated here for the first time. Details of the conjugation mechanism are still to be investigated. The presence of the IS \( \text{sequence: 1245} \) element in the conjugative plasmid \( \text{pMA100} \) is one of the possible molecular mechanisms to explain the distribution of this insertion sequence in mycobacterial species other than \( \text{M. avium} \).

The complete sequencing of this linear plasmid is in progress. Sequence analysis could generate important information about genes involved in the mechanism of horizontal transfer in mycobacteria. Moreover, it has the potential to expand our understanding of how these acquired genes may influence the virulence, evolution and genetic diversity of mycobacteria. Finally, molecular tools could be developed from this conjugative linear plasmid, which could be used in genetic studies in mycobacteria, particularly with the avirulent strain \( \text{M. bovis} \) BCG.

Materials and Methods

Bacterial isolates and strains

Fifteen colonies, named 88.1 to 88.15, used in this study, were isolated from a \( \text{M. avium-M. kansasii} \) mixed culture obtained from a single bone marrow specimen from an HIV-positive patient, as described in a previous study [12]. Four colonies (88.1, to 88.4) were identified as \( \text{M. avium} \) and 11 as \( \text{M. kansasii} \) by phenotypic characteristics (growth rate and pigment production) and by PCR restriction enzyme analysis of the 16S-23S internal transcribed sequence (PRA-ITS) [47] (Table 2). The 11 \( \text{M. kansasii} \) colonies showed indistinguishable PFGE-DraI patterns, except for the presence of a band of approximately 100 kb in eight of the 11 isolates and were, in fact, the same strain [12]. Two \( \text{M. kansasii} \) clinical isolates, IAL 413 and IEC 6805, \( \text{Mycobacterium smegmatis} \) mc\(^{\text{2155}}\), \( \text{Mycobacterium bovis} \) BCG Moreau and \( \text{Escherichia coli} \) DH5\( \text{a} \) (Life Technologies Co., Carlsbad, CA) were used in this study (Table 2).

Mycobacteria were cultivated in Middlebrook 7H9 liquid medium supplemented with oleic acid, albumin, dextrose and catalase (7H9-ADC) (Becton Dickinson, Franklin Lakes, NJ) or in Middlebrook 7H10 solid medium supplemented with oleic acid, albumin, dextrose and catalase (7H10-OADC) and stored at –80 °C until use. Luria-Bertani (LB) liquid or solid medium [48] were used to cultivate \( \text{M. smegmatis} \) mc\(^{\text{2155}}\) and \( \text{E. coli} \). Mycobacterial genomic DNA was extracted from a loop full of culture grown on 7H10-ADC as described by van Soolingen \ et al. [49].

**Table 2. Clinical isolates and strains used in this study.**

| species                  | isolates/strains | PCR-IS 1245 |
|--------------------------|-----------------|-------------|
| \( \text{Mycobacterium avium} \) | 88.1 to 88.4    | +           |
| \( \text{Mycobacterium kansasii} \) | 88.5 to 88.7    | -           |
| \( \text{Mycobacterium kansasii} \) | 88.8 to 88.15   | +           |
| \( \text{Mycobacterium kansasii} \) | IAL 413         | -           |
| \( \text{Mycobacterium smegmatis} \) | IEC 6805        | -           |
| \( \text{Mycobacterium smegmatis} \) | mc\(^{\text{2155}}\) | -           |
| \( \text{Mycobacterium bovis} \) | BCG Moreau      | -           |
| \( \text{Escherichia coli} \) | DH5\( \text{a} \) | -           |

(+) amplification of the 427-bp fragment; (-) no amplification.

doi:10.1371/journal.pone.0029884.t002

PCR-IS 1245

A 427-bp fragment of the IS 1245 element (sequence positions: 197 to 623 – accession number: L33879) was amplified using primers P1 (GCGGGCGAAACGATCTAC) and P2 (AGGTGGCCGTGAGGAAGAC), as described by Guerrero \ et al. [6].

PCR-IS 6110

A 123-bp fragment of the IS 6110 sequence (sequence positions: 762 to 883 – accession number: X17348) was amplified using primers IS1 (CCTGGCGACGTAGGCGTCG) and IS2 (CTCGTCCACGGCGCTTGGG) as described by Eisenach \ et al. [50].

PCR-pMA100

A 206-bp fragment of the sequenced region of pMA100 (sequence positions: 3029 to 3235 – accession number:J560941) was amplified with primers pMA100-1 (ACTCTCGGCTT-CCTGTGTTTG) and pMA100-2 (AGCGAAGTCCACCTTG-GATG) with the same PCR conditions used to amplify a fragment of the IS 1245 element.

Pulsed-field gel electrophoresis (PFGE)

High molecular weight DNA was prepared as described by Viana-Niero \ et al. [51] with some modifications. Bacteria were cultivated in 7H9-ADC at 37 °C to an OD\(_{500}\) of 1–2. Cells were pelleted and frozen at –80 °C for 1 h. After thawing, pellets were suspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 50 mM EDTA) with 0.1% Tween 80. The suspension was mixed with an equal volume of 1% low-melting-point agarose (Bio-Rad Laboratories Inc., Hercules, CA) in 125 mM EDTA pre-warmed to 55 °C, and the mixture was cast into plug molds. The plugs were treated overnight with 2 ml of 10 mg.m\(^{-1}\) lysozyme in STE and incubated at 4 °C for 1 h in 0.5 M EDTA plus 1% Sarkosyl. Proteinase K (RBC Biocience Co., Taipei, Taiwan) was added at a final concentration of 2 mg.ml\(^{-1}\), and the plugs were incubated at 55 °C for 24 h and then at 4 °C for 1 h. Plugs were washed with 1X Tris-EDTA (TE; 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubated in 1X TE containing 0.12 mg.ml\(^{-1}\) phenylmethlysulfonyl fluoride (Sigma-Aldrich Corp., St. Louis, MO) for 1 h at 55 °C. Plugs were washed with 1X TE and stored in 0.5 M EDTA at 4 °C until used. Plugs were washed in 0.05 M EDTA and loaded on 1% pulsed-field-certified agarose gels (Bio-Rad) in 0.5 X TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.0). PFGE was carried out in a CHEF-DR III system (Bio-Rad) at 14 °C for 24 h at 6 V.cm\(^{-1}\) with switch times of 1.6 to 21.3 s. Bacteriophage lambda ladder PFG marker (New England BioLabs, Ipswich, MA) was used as molecular standard. For digestion, plugs were extensively washed in TE and incubated with Dral (Promega, Madison, WI) at 37 °C overnight.

Southern blot hybridization

After PFGE, DNA was blotted onto nylon membranes (Hybond-N-plus; GE Healthcare, Little Chalfont, UK) and probed with an IS 1245 complementary probe, prepared by PCR with primers P1/P2 with DNA from \( \text{M. avium} \) ATCC 25291\(^1\) and purified with QiAquick PCR Purification kit (Qiagen, Valencia, CA), and labeled with \( [\text{x-32P} ] \text{dCTP} \) with Ready-To-Go DNA Labeling Beads (GE Healthcare), according to manufacturers’ instructions.

Characterization of pMA100

Experiments were carried out to determine if the pMA100 molecule was linear or circular. First, its migration in PFGE under three different switch times, 1.6 to 21.3 s, 1 to 30 s, and 10 to 50 s, was evaluated [52].

Besides that, the pMA100 band excised from PFGE agarose gels was treated in separate experiments with 30 U exonuclease III;
30 U exonuclease lambda or 30 U topoisomerase I (all enzymes from New England BioLabs) for 5 h at 37 °C, according to the manufacturer's protocols. The reactions were stopped by adding 50 mM EDTA and cooling to 4 °C for 15 min, and the results were evaluated by PFGE. In separate experiments, proteinase K was omitted during preparation of the plugs to investigate whether pMA100 contained a covalently bound protein. DNA treated or not with proteinase K was subjected to PFGE or to sodium dodecyl sulfate (SDS)-PFGE, i.e., after adding SDS (final concentration 0.2%) to both buffer and agarose gel preparation.

Cloning and sequencing of KpnI restriction fragments containing the IS1245 element

DNA from *M. kansasii* colony 88.8 was digested with KpnI at 37 °C for 2 h, and subjected to RFLP-IS1245, performed as described by van Soolingen et al. [49]. The sizes of fragments hybridizing with the IS1245 probe were calculated using the BioNumerics program version 5.1 (AppliedMaths, Sint-Martens-Latem, Belgium). Agarose gel slices containing the selected KpnI bands were excised from a second gel, and DNA purified using the Gel Extraction Kit (Qiagen), and ligated to pBluescript SK+ (Stratagene, Santa Clara, CA) digested with KpnI and dephosphorylated (CIAP; Life Technologies). Competent *E. coli* DH5α cells were transformed with the ligation products, and recombinant plasmids were selected on LB-agar plates containing 100 μg ml⁻¹ ampicillin, 0.25 mM IPTG and 1 mM X-Gal (Life Technologies). Colony lifts were carried out on Whatman 541 filters and were hybridized with the IS1245-derived probe, labeled with [α-³²P]dCTP, according to the protocol described by Maas [53]. *E. coli* DH5α transformed with the plasmid pMA12, which contains the 427-bp fragment of IS1245, was used as the positive control of hybridization [49].

Recombinant plasmids were purified from positive colonies, identified after exposure to autoradiographic films, using Qiaprep Spin miniprep kit (Qiagen), and the inserts were sequenced using M13 primers and BigDye Terminator Cycle Sequencing Ready Reaction kit (Life Technologies) in an automated ABI Prism 377 sequencer (Life Technologies). Sequences were compared against the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/blast).

The obtained sequence was deposited under GenBank accession number JN560941.

Mating experiments

Mating experiments were performed using *M. avium* 88.3 from the *M. avium-M. kansasii* mixed culture, bearing pMA100, as donor. Three *M. kansasii* strains, colony 88.6 (isolated from the mixed culture), IEC 6805 and IAL 413, *M. smegmatis* mc²[155], and *M. bovis* BCG Moreau were used as recipients. All recipient strains were negative for the presence of both IS1245 and pMA100, as assessed by PCR-IS1245 and Southern blot hybridization of pulsed-field gels with the IS1245 derived probe, respectively.

Mating experiments were carried out on 7H10 agar plates as described by Parsons et al. [54] with some modifications. Donor and recipient strains cultures in 7H-ADC liquid medium were incubated on a shaker at 37 °C until OD600~1.2 was reached. Aliquots of 0.5 ml of each culture were centrifuged at 14,000 x g for 2 min and pellets were suspended in 0.5 ml of 7H9-ADC. Each mating pair suspension was combined and filtered through sterile 0.45 μm membranes (Millipore, Billerica, MA). These filters were incubated on 7H10-OADC plates at 37 °C or 30°C. After different mating periods, the filters were transferred to sterile containers and washed with 7H9-ADC. Bacteria were resuspended in 1 ml of 7H9-ADC, and the suspensions were diluted and plated on 7H10-OADC (when *M. kansasii* or *M. bovis* BCG was used as the recipient strain) or LB (when *M. smegmatis* was the recipient strain) agar plates to obtain isolated colonies.

No selective medium was used to screen transconjugants, because no selection marker has been identified so far in pMA100, and a mixture of donor, recipient and transconjugant colonies was recovered on the 7H10-OADC plates. Only isolated colonies showing the characteristics of the recipient strain were used for screening of transconjugants by PCR-IS1245. *M. kansasii* colonies were identified by the production of yellow pigment after exposure to light. *M. bovis* BCG colonies were identified by PCR-IS66110. When *M. smegmatis* was used as recipient, the mating mixture was plated on LB agar, where *M. avium* cannot grow, and only *M. smegmatis* colonies were recovered.

Recipient colonies that generated IS1245 amplicons after the mating experiments were assumed to be transconjugants.

The presence of plasmid pMA100 and IS1245 in transconjugants was confirmed by PFGE and hybridization with the IS1245-derived probe and with a probe derived from the pMA100 sequenced region, labeled with [α-³²P]dCTP. Each transconjugant was compared to the corresponding wild type strain by PFGE with Dral digested DNA.

Acknowledgments

We thank Erica Chimara and Maria Luiza Lopes for providing *M. kansasii* clinical strains and Cristina Viana-Niero, Monica Vieira Midoli, Ana Carolina de Mello Santos, and Jesus Yubero for excellent technical assistance. Dr. A. Leyva helped with English editing of the manuscript.

Author Contributions

Conceived and designed the experiments: SCL RMS MJG. Performed the experiments: MCSR RSIO CKM. Analyzed the data: LGPdA. Contributed reagents/materials/analysis tools: RSO LGPdA MJG SCL. Wrote the paper: MCSR SCL.

References

1. Mahillon J, Chandler M (1998) Insertion sequences. Microbiol Mol Biol Rev 62: 725–774.
2. Guilhot C, Jackson M, Giopiet B (1999) Mobile genetic elements and plasmids: tools for genetic studies. In: Ratledge C, Dale J, eds. Mycobacteria: molecular biology and virulence. Oxford: Blackwell Science Ltd. pp 17–37.
3. Tortoli E, Rindi L, Garcia MJ, Chiaradonna P, Dei R, et al. (2004) Proposal to elevate the genetic variant MAC-A, included in the *Mycobacterium avium* complex, to species rank as *Mycobacterium chimaera* sp. nov. Int J Syst Evol Microbiol 54: 1277–1285.
4. Murcia MI, Tortoli E, Menendez MC, Palenque E, Garcia MJ (2006) *Mycobacterium chimaera* subsp. *chimaera*, a novel member of the *Mycobacterium avium* complex and description of MAC-X as a new ITS genetic variant. Int J Syst Evol Microbiol 56: 2049–2054.
5. Inderlied CB, Kemper CA, Bermudez LE (1995) The *Mycobacterium avium* complex. Clin Microbiol Rev 6: 266–310.
6. Guerrero C, Bernascoci C, Burki D, Bodmer T, Telenzi A (1995) A novel insertion element from *Mycobacterium avium*, IS1245, is a specific target for analysis of strain relatedness. J Clin Microbiol 33: 304–307.
7. Mijl W, de Haas P, Bossuyt R, Van der Laan T, Rigouts L, et al. (2002) Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subspecies for bird-type isolates and *M. avium* subspecies *hominis* for the human/porcine type of *M. avium*. Int J Syst Evol Microbiol 52: 1505–1518.
8. Oliveira RS, Sírcili MP, Oliveira EMD, Balian SC, Ferreira-Neto JS, et al. (2003) Identification of *Mycobacterium avium* genotypes with distinctive traits by combination of IS1245-RFLP and restriction analysis of krg67. J Clin Microbiol 41: 44–49.
9. Peral-Carmon M, Arbid RD (1998) Characterization of IS1245 for strain typing of *Mycobacterium avium*, J Clin Microbiol 36: 1859–1863.
10. Ritacco V, Kremer K, van der Laan T, Jepinburg JE, de Haas PE, et al. (1998) Use of IS901 and IS1245 in RFLP typing of *Mycobacterium avium* complex:
relatedness among serovar reference strains, human and animal isolates. Int J Tuberc Lung Dis 2: 242–251.
11. Keller AP, Bergg ML, Anholt B, Frans F, Meiners P, et al. (2002) Evidence of the presence of IS1245 and IS1311 or closely related insertion elements in non-tuberculous mycobacteria outside of the Mycobacterium avium complex. J Clin Microbiol 40: 1869–1872.
12. da Silva Rabello MC, de Oliveira RS, Silva RM, Leao SC (2010) Natural occurrence of horizontal transfer of Mycobacterium avium-specific insertion sequence IS1245 to Mycobacterium kansasii. J Clin Microbiol 48: 2257–2259.
13. Gutierrez MC, Brise S, Brosch R, Fabre M, Omais B, et al. (2005) Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. PLoS pathogens 1: e5.
14. Hatfull GF, Jacobs WR, Jr. (1994) Mycobacteriophages: cornerstones of mycobacterial research. In: Boloom BR, ed. Tuberculosis: Pathogenesis, Protection and Control. Washington, DC: ASM Press. pp 163–183.
15. Le Danter C, Winter N, Gicquel B, Vincent V, Picardeau M (2001) Genomic sequence and transcriptional analysis of a 23-kilobase mycobacterial linear plasmid: evidence for horizontal transfer and identification of plasmid maintenance systems. J Bacteriol 183: 2157–2164.
16. Stinear TP, Pryor MJ, Porter JL, Cole ST (2005) Functional analysis and annotation of the virulence plasmid pHU001 from Mycobacterium ulcerans. Microbiology 151: 683–692.
17. Coleman NV, Spain JC (2003) Distribution of the coenzyme M pathway of epoxide metabolism among ethene- and vinyl chloride-degrading Mycobacterium strains. Appl Environ Microbiol 69: 6041–6046.
18. Picardeau M, Vincent V (1997) Characterization of large linear plasmids in mycobacteria. J Bacteriol 179: 2753–2756.
19. Picardeau M, Vincent V (1998) Mycobacterial linear plasmids have an inversion-like structure related to other linear replicons in actinomyces. Microbiology 144 (Pt7): 1981–1986.
20. Bauer J, Andersen AB (1999) Stability of insertion sequence IS1245, a marker for differentiation of Mycobacterium avium strains. J Clin Microbiol 37: 442–444.
21. Sakaguchi K (1990) Invertrons, a class of structurally and functionally related gene families that includes linear DNA plasmid, transposable elements, and genomes of adenovirus-type viruses. Microbiol Rev 34: 66–74.
22. Chen CW, Yu TW, Lin YS, Kieser HM, Hopwood DA (1993) The conjugative plasmid SP2 of Strepotrophus lividus is a 50 kb linear molecule. Mol Microbiol 7: 685–692.
23. Hosted TJ, Wang T, Horan AC (2004) Characterization of the Strepotrophus lactisolate DM1455 linear plasmid pLV15. Microbiology 150: 1819–1827.
24. Kersulyte D, Mukhopadhyay AK, Shirai M, Nakazawa T, Berg DE (2000) Non-conventional conjugal DNA transfer between Streptomyces lividans Rm5 and Rhodococcus sp IMRU 3455 linear plasmid pSLV45. Microbiology 150: 1819–1827.
25. Fang Z, Doig C, Morrison N, Watt B, Forbes KJ (1999) Characterization of Mycobacterium ulcerans strain D2. J Bacteriol 181: 1021–1024.
26. Soby S, Kirkpatrick B, Kosuge T (1993) Characterization of an insertion sequence IS53 located within IS610 on the iaa-containing plasmid of Pseudomonas syringae pv. phaseolicola. Plasmid 28: 135–141.
27. Margueron S, Middleton AT, Wheatcroft R (1995) Characterization, nucleotide sequence, and conserved genomic locations of insertion sequence IS605 in Rhizobium meliloti. J Bacteriol 177: 3133–3142.
28. Blattner FR, Plunkett G, 3rd, Bloch CA, Perna NT, Burland V, et al. (1997) The complete genome sequence of Escherichia coli K-12. Science 277: 1453–1462.
29. Marcia MI, Garcia MJ, Ová I, Gomez AB, Menendez MC (2007) Molecular features of Mycobacterium avium human isolates carrying a single copy of IS1245 and IS1311 per genome. FEBS Microbiol Letters 272: 229–237.
30. Gamieldien J, Ptitsyn A, Hide W (2002) Eukaryotic genes in Mycobacterium tuberculosis could have a role in pathogenesis and immunomodulation. Trends Genet 18: 5–8.
31. Kinsella RJ, McInerney JO (2003) Eukaryotic genes in Mycobacterium tuberculosis? Possible alternative explanations. Trends Genet 19: 687–689.
32. Podzareks GJ, Kalakov LA, Larkin MJ, van Wykhamm Vleg JE, Janssen DB (2000) Roles of horizontal gene transfer and gene integration in evolution of 1,3-dichloropropene- and 1,2-dibromoethane-degradative pathways. J Bacteriol 182: 2191–2199.