INSERTION SEQUENCE IS*1141*: DISCOVERY, CHARACTERIZATION, AND ASSOCIATION WITH *MYCOBACTERIUM INTRACELLULARE* COLONIAL VARIATION

by

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(ABSTRACT)

Mycobacterium avium and *Mycobacterium intracellulare*, (*M. avium* complex, MAC) are human pathogens causing disease in individuals with acquired immunodeficiency syndrome (AIDS) or with thoracic abnormalities. MAC bacteria are difficult to kill because of the resistance of the pathogens to chemotherapeutic agents. One factor affecting treatment of MAC disease is the presence of interconvertible colonial variants. Transparent (T) variants have greater resistance to antibiotics and higher pathogenicity; opaque (O) variants are more susceptible to antibiotics and less pathogenic. The overall goal of this study was to investigate the mechanism for colonial variation. Based on an observation that T variants of *M. intracellulare* strain Va14 contained a plasmid which was 6 kb smaller than the 68 kb plasmid in O variants, it had been suggested that a transposable element might be responsible for colonial variation.

The first objective was to clone the unique DNA fragment present in the 68 kb plasmid but absent from the 62 kb plasmid. The second and third objectives were to determine if the unique fragment contained a transposable element and to analyze the role of that element in the mechanism of colonial variation in *M. intracellulare* strain Va14. The fourth objective was to determine the distribution of IS*1141* in MAC isolates.

Fragments containing copies of the putative element were sequenced and a region 1596 basepairs in length with 23 basepair imperfect inverted repeats was designated as insertion sequence IS*1141*. IS*1141* is the first insertion sequence identified in *M. intracellulare*. Data base searches using open reading frames (ORF) of IS*1141*, identified ORFb as significantly similar to the transposases of the IS*3* family. The presence or absence of IS*1141* in strain Va14 plasmids appeared unrelated to colonial variation, but IS*1141* was present in another plasmid and the chromosome of the Va14 variants. Hybridization studies with IS*1141* identified three chromosomal copies in O variants and two chromosomal copies in T variants. Va14 T variants each had a common IS*1141* restriction fragment length polymorphism (RFLP) pattern which was different than the single RFLP pattern found in opaque variants. Based on these differences, it appears that IS*1141* may integrate into the gene(s) responsible for the T phenotype preventing their expression. A survey of 64 James River basin non-AIDS, clinical and James River environmental MAC isolates identified 4 of 24 (17%) *M. intracellulare* isolates as containing IS*1141*. IS*1141* has not been detected in any clinical or environmental *M. avium* or *Mycobacterium* species X isolates and may be limited to *M. intracellulare*.

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Graduate school has been much like an extended, working vacation to the circus; a circus has both exciting and frightening moments, exhilarating rides, and sights and smells which sometimes generate nausea. My time in the Biology Department has been filled with excitement, disappointment, and even a little nausea. This "vacation" has been shared with some very special people which I would like to acknowledge.

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CHAPTER I

Introduction

Members of the *Mycobacterium avium* complex (MAC) include *Mycobacterium avium* subspp. *avium* and *silvaticum*, *Mycobacterium intracellulare*, *Mycobacterium lepraemurium*, and *Mycobacterium paratuberculosis* (Thorel *et al.*, 1990; Wayne *et al.*, 1991; Wayne and Sramek, 1992). *M. paratuberculosis* can be distinguished from the other MAC because it requires media supplemented with iron-chelating mycobactin and *M. lepraemurium* is distinguished by its failure to grow on most mycobacteria media. *M. avium* subspecies and *M. intracellulare* can not be distinguished by traditional biochemical tests, but most *M. avium* isolates are more susceptible to sodium nitrate than are *M. intracellulare* isolates (Sato *et al.*, 1992). Isolates of *M. paratuberculosis* and *M. avium* subsp. *silvaticum* can be distinguished by the presence of the insertion sequences IS*900* (Green *et al.*, 1989) and IS*902* (Moss *et al.*, 1992) respectively, but no specific insertion sequence (IS) has been associated with *M. avium* subsp. *avium* or *M. intracellulare*. The development of species specific DNA probes (Accu-Probe, Gen-Probe, Inc. San Diego, CA; SNAP System, Syngene, Inc., San Diego, CA) has greatly simplified identification of *M. avium* and *M. intracellulare* isolates.

M. avium complex organisms are present in the surface waters (Falkinham *et al.*, 1980; Kirschner *et al.*, 1992), aerosols (Wendt *et al.*, 1980), and soils (Brooks *et al.*, 1984; Kirschner *et al.*, 1992) of the eastern United States. MAC organisms have been recovered from municipal (duMoulin and Stottmeier, 1986) and hospital (duMoulin *et al.*, 1988) water systems, but a specific environmental source for human pathogenic strains has not been identified. Evidence for an environmental source for MAC pathogens includes; (1) MAC isolates from the environment share plasmids of similar sizes (Meissner and Falkinham, 1986) and homology groups (Jucker and Falkinham, 1990) with clinical isolates, (2) *M. avium* soil

isolates share similar random amplified polymorphic DNA profiles with acquired immunodeficiency syndrome (AIDS) patient isolates (Via *et al.*, in preparation), (3) and MAC strains share phenotypic characters with clinical strains (Fry *et al.*, 1986). In addition, there has been no evidence of person-to-person transmission of MAC bacteria as is seen in *Mycobacterium tuberculosis* complex infections (Wolinsky, 1992).

M. avium complex organisms have been isolated from wild and domesticated birds and from most mammals including livestock species and humans (Grange *et al.*, 1990). Although MAC organisms are frequently isolated from healthy animals (Grange *et al.*, 1990) and from stools of apparently healthy humans (Wolinsky, 1992), these organisms potentially cause three types of disease: localized primary lymphadenitis, pulmonary infection, and disseminated disease (Wolinsky, 1992).

Lymphadenitis usually occurs in the cervical nodes of immunocompetent children; disease originates by oral pathogen entry (Wolinsky, 1979). Although this disease was originally associated with *M. scrofulaceum* in the U.S.A., the majority of cases treated today are caused by MAC bacteria (Wolinsky, 1992).

Pulmonary disease due to *M. avium* complex organisms is similar to tuberculosis in its range of severity. The majority of cases occur in immunocompetent individuals with chronic obstructive lung disease or predisposing factors such as abnormal thoracic cavity shape, reduced lung capacity, reduced ciliary activity in the lung (Iseman, 1989), or advanced age. Diagnoses of disease caused by MAC organisms in middle age women with no obvious predisposing condition are increasing (Iseman, 1989). The present rate of non-AIDS related MAC cases is 1.2 per 100,000 per year with 50% of infection caused by *M. avium* and 50%

by *M. intracellulare* (Wolinsky, 1992).

In recent years, AIDS has emerged as a major predisposing condition for disease caused by *M. avium* complex bacteria. Ellner *et al.* (1991) report *M. avium* has been detected in 18 to 27% of living human immunodeficiency virus-(HIV) seropositive patients with CD4 counts below 50 and in 47 to 53% of autopsied AIDS patients. Studies by Horsburgh *et al.* (1991) show that untreated MAC disease reduce the life expectancy of AIDS patients by approximately 5 months (*i.e.*, one-half their expected survival). *M. avium* strains with serotypes 1, 4, or 8 are recovered from 97% of AIDS patients with MAC disease (Ellner *et al.*, 1991); rarely is *M. intracellulare* recovered (Wolinsky, 1992).

M. avium complex bacteria, like *Neisseria gonorrhoeae* and *Salmonella typhimurium*, produce colony or phase variants differing in pathogenicity. Dunbar *et al.* (1968) observed that MAC isolates form two types of smooth variants, smooth domed opaque (O) and smooth thin transparent (T), as well as a rough variant (R). While R variants produce only R derivatives (McCarthy, 1970), the smooth O and T variants segregate variants of the opposite morphology at high frequency; thus, the O and T colonial variants are interconvertible. Transition from the transparent to the opaque colony form occurs at a rate of 10^{-4} to 10^{-5} per generation (McCarthy, 1970), while the conversion from opaque to transparent colony form occurs at a rate of 10^{-6} to 10^{-7} per generation (Woodley and David, 1976).

Individual *M. avium* complex strains differ in virulence in chickens and mice, but opaque variants of a strain are generally less virulent than their transparent parents (Schaefer *et al.*, 1970; Kuze and Uchihira, 1984). Animal isolates initially contain predominately T colony-formers but with continued laboratory subculture, the O variant becomes dominant

(Dunbar *et al.*, 1968). Isolates from immunocompetent patients are normally T colony formers whereas isolates from AIDS patients can be T, O, or of mixed colony forms (D. Dawson, personal communication), illustrating that the weakened immune system of some AIDS patients is ineffective in removing even the less virulent colony forms of MAC bacteria.

M. avium complex bacteria are found as intracellular parasites in monocytes and macrophages. Opaque variants are more readily phagocytized by monocytes than the T variant of the same strain (Shiratsuchi *et al.*, 1990). Once in the phagosome, T variants appear to inhibit phagosome-lysosome fusion and proliferate in the cell (Kallenius *et al.*, 1992). Opaque variants are less likely to reproduce in the monocytes and may be killed in lysosome-fused vacuoles (Micheleini-Norris *et al.*, 1992). Monocytes which have engulfed O variants release much higher levels of immune system-stimulating IL-1β and IL-6 than do monocytes harboring the isogeneic T variant (Micheleini-Norris *et al.*, 1992). In some monocyte samples, T-infected cells produce little more than background, levels of cytokines IL-1α and IL-1β *in vitro* (Micheleini-Norris *et al.*, 1992). Adding to the problems of HIV-infected immune system, HIV-1 infected monocytes produce and secrete lower levels of IL-1 upon *M. avium* infection than non-HIV-1 infected monocytes (Kallenius *et al.*, 1992). The cell surface of the T bacterium may be responsible for its ability to stimulate minimally the normal host immune system, while the O variant activates the immune system.

M. avium complex organisms contain polysaccharides and C-mycosides in a outer cell wall layer thought to be responsible for their general antibiotic resistance (Rastogi *et al.*, 1981). David *et al.* (1987) observed that the outer cell wall layer of T variants was thick and continuous but that the layer was discontinuous in O variants. Thorel and David (1984) found

antigens produced in T variants but not in O variants. Opaque colonial variants are more susceptible to aminoglycosides, cephalosporins and rifampicin than the more virulent T and rough variants (Schaefer, 1970; Mizuguchi *et al.*, 1983; Kuze and Uchihira, 1984) suggesting that O variants may be more permeable to these drugs than their T counterparts.

Transparent variants grow more slowly in Middlebrook 7H9 medium than do their opaque derivatives (Kajioka and Hui, 1978) unless high concentrations of Tween 80 are added to the medium. Transparent variants require a source of fatty acids for growth (Kajioka and Hui, 1978) and are more resistant to high concentrations of fatty acids than are their O derivatives (Saito and Tomioka, 1988). The differences in T and O colonial variants growth requirements, antibiotic sensitivity, and virulence have been characterized (Table 1.1), but the genetic mechanism responsible for colonial variation and its associated change in virulence has not been resolved.

In other bacteria, mechanisms of phase variation include site-specific inversion of a regulatory DNA region (Glasgow *et al.*, 1989), duplicative transposition of genes into a site of expression (gene conversion; Swanson and Koomey, 1989), deletion of intervening DNA between regulatory regions and antigen-coding genes (Swanson and Koomey, 1989), precise insertion or deletion of DNA sequences, and DNA rearrangement (Glasgow *et al.*, 1898). Gene amplification has also been implicated in colonial hypervariability in *Streptomyces ambofaciens* (Le Blond *et al.*, 1989).

Table 1.1. Summary of observed differences in opaque and transparent *M. avium* complex variants.

Characteristic	Opaque	Transparent	Reference
Variation rate to opposite morphology (variants/cell/generation)	$10^{-4 \text{ to } -5}$	$10^{-6 \text{ to } -7}$	McCarthy, 1970 Woodley and David, 1976
Virulence of variants			
Animals	avirulent	virulent	Schaefer *et al.*, 1970 Kuze and Uchihira, 1984
Immunocompetent humans	avirulent	virulent	Wolinsky, 1992
AIDS patients	virulent	virulent	D. Dawson, 1989 Kallenius *et al.*, 1992
Monocyte-Macrophage response to variants			
Phagocytosis	readily	reduced	Shiratsuchi *et al.*, 1990
Bacterial killing	little	none	Micheleini *et al.*, 1992
Bacterial growth inhibition	yes	none	Kallenius *et al.*, 1992
Cytokine production	stimulated	minimal	Micheleini *et al.*, 1992
Antibiotic susceptibility	sensitive	resistant	Schaefer *et al.*, 1970 Kuze and Uchihira, 1984
Generation time	16 to 18 h	24 to 26 h	Woodley and David, 1976
Fatty acid requirement	none	yes	Saito and Tomioka, 1988
Variant-specific antigens	none	yes	Thorel and David, 1984

Previously suggested colonial variation mechanisms for MAC organisms include (1) lysogeny (Jones and White, 1967), (2) mutation (McCarthy, 1970; Woodley and David, 1976), and (3) the loss of a 3 kD plasmid (Mizuguchi *et al.*, 1981). Some of these mechanisms were proposed before the interconvertible nature of O and T variants was demonstrated. If mycobacteriophage were responsible for the observed interconversion from rough to smooth colonies (Jones and White, 1967), cells isolated away from the phage would not be capable of interconversion and strains locked in one colonial form when reinfected with phage should gain the ability to change colonial morphology again. This possibility has not been investigated. The rates of colonial variation are far higher than mutation rates (McCarthy, 1970; Woodley and David, 1976) and T and O variants are interconvertible (Woodley and David, 1976). Plasmid loss is probably not responsible (Mizuguchi *et al.*, 1981) because reversion would not be possible. Integration of the 3 kD plasmid into the chromosome was not investigated due to strain loss.

Recently, a 22 kilobase (kb) deletion in the gene cluster responsible for the serotype 2 glycopeptidolipids (*Ser*2) in an *M. avium* serovar 2 strain has been cited as responsible for the smooth to rough transition of a rough variant (Belisle *et al.*, 1991). This rough variant is unable to synthesize some of the surface glycolipids. Since the difference in O and T variants is believed to be differences in the cell surface glycopeptideolipids (David *et al.*, 1987), it is possible that the *Ser*2 DNA region might also be the DNA region involved in colonial variation. Transformation of the intact *Ser*2 gene cluster into *Mycobacterium smegmatis* enabled the transformants to produce some serotype 2 antigens (Belisle *et al.*, 1991), but no O colonies or T colonies have been observed (Jonathan Mills, personal communication).

An observation that T variants of *M. intracellulare* strain Va14 contained a plasmid which was 4 to 6 kb smaller than the 68 plasmid in opaque variants (Erardi *et al.*, 1985) suggested that transposition of a mobile genetic element might be responsible for colonial variation in *M. avium* complex, but no mycobacterial transposons (Tn) or insertion sequences had been identified at that time.

The IS elements identified in slow-growing mycobacteria have been of two types: typical and atypical. The *M. tuberculosis* element IS*6110*, also called IS*986,* (Thierry *et al.*, 1990; McAdam *et al.*, 1990) and IS*1081* (Collins and Stephens, 1991) from *Mycobacterium bovis* are typical insertion sequences containing terminal inverted repeats flanked by short direct repeats. The elements are present in the genome of their hosts in multiple copies which vary in position with strain. The IS*6110* large open reading frame (ORF) has some similarity to the putative IS*3411* transposase (McAdam *et al.*, 1990) from the citrate utilization transposon Tn*3411* (Ishiguro and Sato, 1988). IS*3411* is a member of the IS*3* family whose members are found in *Escherichia coli* and *Shigella* species. The atypical IS elements lack terminal inverted repeats and direct repeats of the insertion site. These closely related elements, IS*900* from *M. paratuberculosis* (Green *et al.*, 1989), IS*901* from *M. avium* (Kunze *et al.*, 1991), and IS*902* from *M. avium* subsp. *silvaticum* (Moss *et al.*, 1992), appear to have rather specific target insertion sites which are regenerated once the IS element is inserted (Kunze *et al.*, 1991). These *M. avium* complex IS elements share target insertion sequence similarity with IS*116* from *Streptomyces clavigerus* and significant amino acid sequence similarity among their putative transposases (Kunze *et al.*, 1991).

The only transposable element reported to be associated with a phenotype is the

sulphonamide-resistance transposon, Tn*6100*, identified in a plasmid of *Mycobacterium fortuitum* (Martin *et al.*, 1990). The transposase of Tn*6100* shares similarity with the integrase gene of Tn*1696* a member of the Tn*21* family from *E. coli*. A Tn*6100*-pUC18 construct which was unable to replicate as a plasmid in *M. smegmatis* has been shown to transpose after transformation into a competent *M. smegmatis* strain (Martin *et al.*, 1990). Neither Tn*6100* nor the mycobacterial IS elements have been reported to be associated with changes in MAC colonial morphology.

The presence of two interconvertible colonial variants makes treatment of MAC disease more difficult. Transparent variants have greater resistance to antibiotics and higher pathogenicity; O variants are more susceptible to antibiotics and less pathogenic. Based on the observation that T variants of *M. intracellulare* strain Va14 contained a plasmid which was 6 kb smaller than the 68 plasmid in O variants, Erardi *et al.* (1985) suggested that a transposable element might be responsible for colonial variation in *M. avium* complex. The major objective of the this study was to investigate the mechanism of colonial variation in *M. avium* complex. The investigation described in this dissertation was initiated with the hypothesis that the 4 to 6 kb DNA fragment present in the 68 kb plasmid in the O variant of *M. intracellulare* strain Va14 was responsible for colonial variation.

Main Objectives

1. Develop a method to isolate and clone the 6 kb unique region of the *M. intracellulare* strain Va14 68 kb plasmid.

2. Determine if the 6 kb unique region of *M. intracellulare* strain Va14(O) 68 kb plasmid contained a transposable genetic element by restriction mapping and sequencing. Analyze the role of that genetic element in colonial variation.

3. Determine the distribution of the transposable element in *M. avium* complex bacteria isolates.

CHAPTER II

Isolation of Restriction Fragments from Large Plasmids Recovered from Bacteria with Multiple Plasmids

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ABSTRACT

A rapid and simple method for isolation of DNA restriction fragments from large plasmids is described. The loss of large plasmids is avoided by restriction endonuclease cleavage in an agarose gel before DNA precipitation. Plasmids were separated in low-melting-point agarose by electrophoresis; the desired plasmid DNA band was cut from the gel and digested with a restriction endonuclease in the agarose. Restriction fragments in agarose were recovered by a modified phenol-extraction, concentrated with 2-butanol and precipitated with ethanol. The procedure simplifies the task of cloning genes from large plasmids, resulting in high yields of restriction fragments from a desired plasmid in a short time.

INTRODUCTION

In a number of bacteria, large plasmids have been shown to encode for pathogenicity [*e.g., Shigella flexneri* (Hardy, 1986) and *Agrobacterium tumefaciens* (Zambryski *et al.*, 1989)] and in *Mycobacterium scrofulaceum*, a 150 kb plasmid encodes mercury-resistance (Meissner and Falkinham, 1986) and copper-resistance (Erardi *et al.*, 1987). Recently, we have discovered that one of four plasmids in *Mycobacterium intracellulare* strain Va14 (*i.e.*, pVT203) carries genetic determinants influencing colonial variation (Via and Falkinham, 1991a). However, recovery of pVT203 using electroelution into troughs (Le Brun *et al.*, 1988), (Electro-eluter, Bio-Rad, Richmond, CA), "freeze and squeeze" (Thuring *et al.*, 1975), the GeneClean™ kit (BIO101, La Jolla, CA), or phenol extraction from low-melting-point (LMP) agarose (Maniatis *et al.*, 1982) was judged unsuccessful because intact plasmid recovery was less than 5%. Herein, we describe a method for isolation of restriction endonuclease fragments of specific large plasmids. The critical step involves restriction endonuclease cleavage of plasmid DNA before separation from the agarose gel.

MATERIALS AND METHODS

Plasmid DNA Separation. Total plasmid DNA was isolated by a modification of the Kado and Liu method (1981) described by Jucker and Falkinham (1990). Following phenol extraction (Jucker and Falkinham, 1990), the DNA-containing aqueous phase was transferred to microcentrifuge tubes using a large bore pipet to avoid the denatured proteins at the interface. A concentrated tracking dye solution (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 50% glycerol, 0.2% xylene cyanol) was added and the DNA was electrophoresed in a 0.5% LMP agarose gel (prepared in TAE: 40 mM Tris-acetate pH 7.8, 1 mM EDTA; Maniatis *et al.*, 1982). The chilled gel was loaded with the DNA sample before placing it in the electrophoresis chamber (Model H33 "minnie" submarine, Hoefer Scientific, San Francisco, CA). The chamber was filled with TAE such that the edges of the gel were in contact with the buffer without flooding the upper surface of the gel. Once the dye front had entered the gel, additional buffer was added to cover the gel and electrophoresis (5 V/cm) was continued for approximately 2 h.

Following electrophoresis, the gel was soaked in 0.5 μg/ml ethidium bromide and the desired plasmid bands, identified by brief UV illumination, were cut out. The gel slabs (about 250 μl) were soaked in 20 volumes sterile 5 mM Tris-HCl pH 8.0 for 2 h at 22° C to remove inhibitors of restriction endonuclease activity. The liquid was drawn off the gel slab with a pipet and the gel slab was placed in a 1.5 ml microcentrifuge tube.

Plasmid Digestion. Restriction endonuclease digestions were performed in a total volume of 300 μl or less, so that DNA isolation procedures could be completed in a single

microcentrifuge tube. Sterile distilled water was added to bring the gel volume up to 269 µl before melting at 65° C. Once melted, the gel was cooled to the restriction endonuclease's suggested incubation temperature, 30 µl of the appropriate 10X reaction buffer and 10 U or more of the restriction enzyme were added (*i.e.*, 5 to 10 times more enzyme than would normally be indicated by the amount of DNA present). At least a 4 h incubation was required for complete digestion.

DNA Isolation from Agarose. One volume melting buffer (0.1 M Tris-HCl pH 8.0, 2 mM EDTA, 50 mM NaCl) was added to the digest and the mixture was heated at 65° C until the agarose was completely melted. The mixture was cooled to 37° C and extracted with an equal volume of 0.1 M Tris-HCl buffered phenol (pH 8.0) (Maniatis *et al.*, 1982). The phases were separated by centrifugation (10,000 x g, for 5 min at 4° C). The aqueous phase was removed and extracted, in turn, with phenol-chloroform and chloroform-isoamyl alcohol (Maniatis *et al.*, 1982). Following these extractions, the aqueous DNA solution was concentrated by mixing with an equal volume of 2-butanol (Wallace, 1987) and centrifuged (5,000 x g for 1 min at 22° C). The upper butanol layer was discarded and the extraction repeated until the volume was reduced by 50%.

To precipitate the DNA, 0.4 volumes of cold 7 M ammonium acetate and 2.5 volumes of ice-cold 95% ethanol were added. After mixing and incubation in ice for 15 min, the DNA solution was centrifuged (14,000 x g for 15-20 min at 4° C). The pellet was washed with ice-cold 70% ethanol and dried at 22° C (Maniatis *et al.*, 1982) and the DNA resuspended in TE.

RESULTS AND DISCUSSION

To compare the success of the various procedures for isolating DNA from gels, equal samples of pVT203 DNA in agarose gel slices were treated as described in the legend to Figure 2.1. DNA recovery was estimated by ethidium bromide fluorescence quantitation using the minigel method (Maniatis *et al.*, 1982). The limited (<20 ng) recovery of intact plasmid is evident in lanes 2, 3, 4, and 5. Ethanol precipitation of the intact plasmid appeared to result in low recovery. If butanol concentration followed by drop dialysis (Wallace, 1987) was substituted for ethanol precipitation, more DNA was recovered, but it was resistant to cleavage with *Hin*dIII and *Eco*RI (data not shown).

Although DNA recovery improved if pVT203 was digested with a restriction endonuclease before it was extracted from the LMP agarose, not all phenol extraction methods gave the same yield of pVT203 restriction fragments (Fig. 2.1). Equal amounts (400 ng) of uncut pVT203 DNA were used for each of the following isolation procedures and the resulting DNA was suspended in 12 µl TE and 3 µl of that DNA solution was loaded on the gel (Fig. 2.1). Using the Wieslander (Maniatis *et al.*, 1982) (lane 6) or Ogden and Adams (1987) (lane 7) procedures, 150 ng of DNA was recovered. A significantly larger amount of DNA (> 240 ng) was recovered with the method described here (lane 8). In addition to lower DNA recovery, the two published methods had other drawbacks. The Wieslander phenol-extraction procedure (Maniatis *et al.*, 1982) (lane 6), resulted in too large a liquid volume for a single microcentrifuge tube. The extraction procedure of Ogden and Adams (1987) (lane 7), resulted in salt precipitation if the DNA solution was concentrated with butanol before ethanol addition.

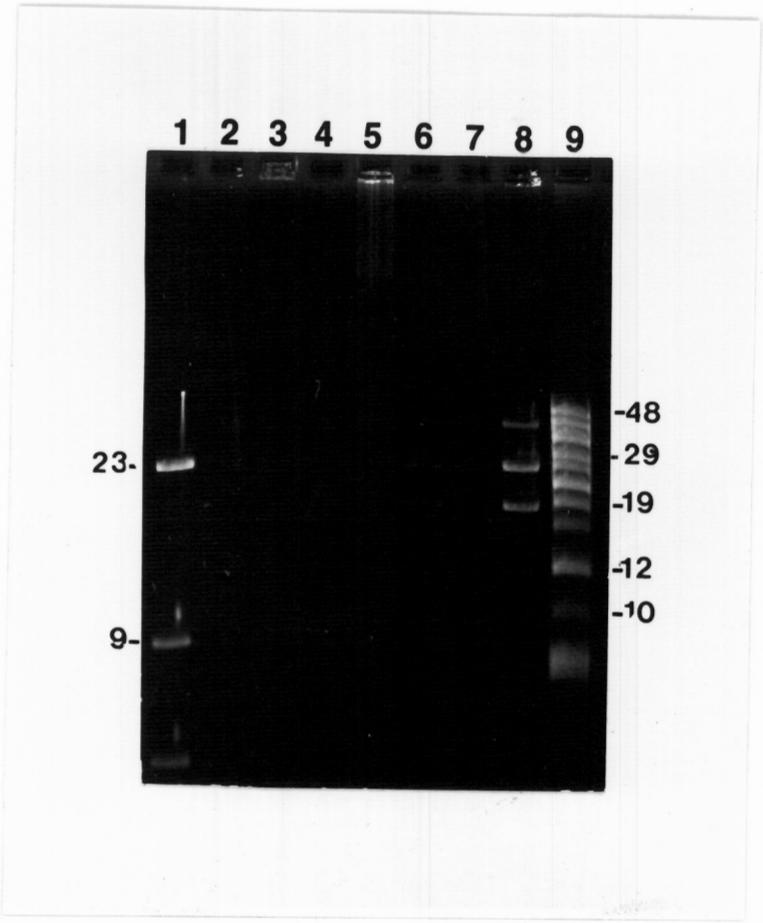

FIG. 2.1. Comparison of methods for recovery of large plasmids. Lane 1: 200 ng *Hin*dIII digested lambda DNA. Lane 2: Electro-eluter (Bio-Rad, Richmond, CA) isolated DNA; lane 3: GeneClean kit (BIO 101, La Jolla, CA) isolated DNA; lane 4: "freeze and squeeze" (Thuring *et al.*, 1975) isolated DNA; and lane 5: DNA extracted from LMP agarose by the Wieslander method (Maniatis *et al.*, 1982). In lanes 6, 7 and 8, the DNA was digested with *Hin*dIII before isolation. Lane 6: DNA prepared using the Wieslander method (Maniatis *et al.*, 1982); lane 7: DNA extracted by the method of Ogden and Adams (1987); and lane 8: DNA extracted as described in this report. Lane 9: 250 ng of high MW DNA markers (Gibco BRL, Life Technologies Gaithersburg, MD).

The procedure described simplifies the task of isolating restriction fragments from large plasmids, resulting in high yields of restriction fragments from a desired plasmid in about 24 hours with few equipment requirements. Restriction fragments from pVT203 isolated by this method have been cloned into pUC19 and pBR322 following published methods (Maniatis *et al.*, 1982).

CHAPTER III

Discovery and characterization of IS*1141* from *Mycobacterium intracellulare* and relationship of IS*1141* to colonial variation.

ABSTRACT

Mycobacterium avium and *Mycobacterium intracellulare*, (*M. avium* complex, MAC) are human pathogens causing disease in acquired immunodeficiency syndrome (AIDS) patients. MAC isolates exist as interconvertible transparent (T) and opaque (O) colonial variants. Transparent variants have greater resistance to antibiotics and higher pathogenicity; O variants are more susceptible to antibiotics and less pathogenic. The *M. intracellulare* transparent strain Va14(T) contained plasmids of 240, 62, 25, and 16 kb. Opaque variants of *M. intracellulare* Va14(T) arose at a rate of 10^{-4} variants/cell/generation and contained 4 plasmids (240, 68, 25, and 16 kb). Transparent variants of one Va14(O) variant arose at a rate of 10^{-6} to 10^{-7} and two of these variants had the same plasmid profile as the Va14(T) strain (240, 62, 25, and 16 kb), suggesting that a transposable element was involved in the change in the 68 kb plasmid and in colonial variation. The putative transposable element from the 68 kb plasmid was cloned and sequenced. The element, designated IS*1141*, is 1596 bp in length, has 23 bp imperfect inverted repeats, and has an open reading frame (ORFb) whose amino acid sequence is significantly similar to the transposase of IS*911*, a IS*3*-related element, from *Shigella dysenteriae*. When a large number of variants were examined, both T and O variants were found to have a 68 kb plasmid containing IS*1141;* a 16 kb plasmid also contained IS*1141*. In all of seven independently isolated T variants and the parental T strain, IS*1141* hybridized with two (3.4 and 2.2 kb) *Sal*I chromosomal fragments. In all of seven independently isolated O variants and the parental O strain, the element hybridized with three (11, 5.2, and 2.2 kb) *Sal*I chromosomal fragments. Thus, the IS*1141* restriction fragment length polymorphism (RFLP) patterns of *M. intracellulare* strain Va14 O and T variants correlated with a distinct colonial morphology.

INTRODUCTION

Mycobacterium avium and *Mycobacterium intracellulare* (*M. avium* complex, MAC) species are opportunistic human pathogens whose presumed source of inoculum is the environment (Fry *et al.*, 1986; Via *et al.*, in preparation). The susceptibility of AIDS patients to disseminated MAC infections is well documented (Blaser and Cohen, 1986; Ellner *et al.*, 1991). In these patients, MAC infection results in increased morbidity and mortality (Horsburgh *et al.*, 1991). In addition, the rate of non-AIDS related MAC disease is rising (Iseman, 1989) with 50% of infection attributed to *M. avium* and 50% to *M. intracellulare* (Wolinsky, 1992). Unfortunately, antibiotic treatment is often unsuccessful because of the characteristic drug resistance of members of the MAC to anti-tuberculosis drugs (Young, 1988). Little is known of the mechanism(s) of MAC antibiotic resistance beyond the suggestion that there exist antibiotic permeability barriers (Rastogi *et al.*, 1981; Mizuguchi *et al.*, 1983). One factor that may contribute to antibiotic resistance is the emergence of antibiotic-resistant, transparent (T) colonial variants at frequencies of 10^{-6} to 10^{-8} variants/cell/generation. *M. avium* and *M. intracellulare* isolates form transparent or opaque (O) colonies upon primary isolation. However, individual isolates segregate variants of the opposite morphology at high frequency, thus, the O and T colonial types are interconvertible. Transparent variants are antibiotic-resistant (Kuze and Uchihira, 1984), virulent in animal models (Schaefer *et al.*, 1970), and require fatty acids for growth (Kajioka and Hui, 1978), while the O variants are antibiotic sensitive, less virulent, and able to grow without fatty acid supplements. Transition from the T to O occurs at a rate of 10^{-4} to 10^{-5} per generation

(McCarthy, 1970), while the conversion from O to T occurs at a rate of 10^{-6} to 10^{-7} per generation (Woodley and David, 1976).

Neither chromosomal mutation, nor plasmid loss (Mizuguchi *et al.*, 1981) are sufficient to explain colonial variation. The rates of variation (McCarthy, 1970; Woodley and David, 1976) are far higher than predicted mutation rates and plasmid loss would preclude reversion. This investigation was initiated after the observation that T variants of *M. intracellulare* strain Va14 contained a plasmid which was 6 kilobase pairs (kb) smaller than the plasmid in O variants (Erardi *et al.*, 1985). Upon reversion to the original colony type, the original plasmid profile was either restored or a new profile appeared (Via and Falkinham, 1991a). While investigating the DNA rearrangements in this strain, we identified an insertion sequence present in two different plasmids and in the chromosome, whose location is associated with colonial morphology. Both typical and atypical insertion sequences (IS) have been identified in mycobacteria (Green *et al.*, 1989; Thierry *et al.*, 1990; McAdam *et al.*, 1990; Kunze *et al.*, 1991; Collins and Stephens, 1991; Moss *et al.*, 1992). Their location at different sites in the genome suggests these elements may transpose.

This work is the first report of a insertion sequence (IS) in *M. intracellulare* and of a *M. avium* complex IS associated with a phenotype. IS*1141* is 1596 bp in length with 23 bp terminal inverted repeats and putative promoters directed outward at either end. The chromosomal IS*1141* restriction fragment length polymorphism (RFLP) patterns of the Va14 transparent variants were all identical to each other and distinct from the RFLP pattern of observed in each of the Va14 opaque variants. The observations reported here provide evidence for several possible mechanisms of colonial variation.

MATERIALS AND METHODS

Mycobacterial strains. A single *M. intracellulare* non-AIDS, clinical isolate (Va14; obtained from Dr. Nancy Warren, Consolidated Laboratories, Richmond VA) was used. Strain Va14 formed two distinct, interconvertible, colonial variants which appeared as either T or O colonies on certain types of mycobacterial media. Colonial variants of *M.intracellulare* strain Va14(O) (LV1) and Va14(T) (LV2) that were isolated and characterized during the course of this study are listed in Table 3.1. The symbols used to designate variants isolated in this study were as follows: (TO), an opaque isolated from the parental transparent (T); (OT), a transparent isolated from a parental opaque (O); (OTO), an opaque isolated from an OT, and (TOT), a transparent isolated from a TO.

Growth of mycobacteria. Stock cultures of newly isolated variants were incubated at 37° C in Middlebrook (M) 7H9 broth base (BBL. Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin (OA) (Pethel and Falkinham, 1989) [MGE broth] for 4 to 7 d. The stock cultures were stored at -70° C after bringing the broth to 20% (vol/vol) glycerol. For all experiments, the frozen stock cultures were scraped with an inoculating loop and the material was streaked on Middlebrook 7H10 agar containing 5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin (OA) enrichment (Pethel and Falkinham, 1989) [MGE agar]. The plates were sealed with Parafilm (American National Can, Greenwich, CT) and incubated at 37° C for 3 wk. Single isolated colonies of the desired morphology were inoculated into 2 ml of MGE broth and incubated until they reached log phase (4 to 7 d). These 2 ml cultures were used as inoculum in subsequent

Table 3.1. Description of *Mycobacterium intracellulare* strain Va14 variants used in this study.

Bacterial strain	Morphology	Lineage	Plasmid profile kb[a]	Reference
LV1 [Va14-(O)]	Opaque (O)	Parental	240, 68, 25, 16	Erardi *et al*, 1985
LV11	Transparent (T)	OT	62	Via and Falkinham, 1991
LV12	Transparent	OT		This study
LV15	Opaque	OTO		This study
LV17	Opaque	OTO		This study
LV114	Opaque	OTO		This study
LV115	Transparent	OT	62	This study
LV116	Opaque	OTO		This study
LV2 [Va14-(T)]	Transparent	Parental	240, 62, 25, 16	Erardi *et al*, 1985
LV21	Opaque	TO	68	Via and Falkinham, 1991
LV23	Transparent	TOT	68	This study
LV25	Transparent	TOT	68	This study
LV26	Opaque	TO	68, 18	Via and Falkinham, 1991
LV29	Transparent	TOT	68	This study
LV213	Transparent	TOT	68	This study
LV219	Opaque	TO	68	This study

[a] Plasmids in variant strains listed if size (kb) is different from the parental strain.

experiments. All cultures received 0.1 volume inoculum unless otherwise stated.

Isolation of independent colonial variants. Single transparent or opaque colonies were suspended in 2 ml of sterile buffered saline-gelatin [BSG; 0.15 M NaCl, 2 mM KH_2PO_4, 4 mM Na_2HPO_4, 0.1% (wt/vol) gelatin, pH 7.0]. This cell suspension was used to inoculate 10 screw-cap tubes (125 mm X 16 mm) each containing 2 ml of MGE broth. The 10 cultures resulting from this procedure were considered siblings, while those cultures inoculated with different colonies were considered independent cultures. Variant LV11, showing reversion from opaque to transparent (OT), was used to isolate opaque (OTO) derivatives and variant LV21, a transparent to opaque (TO) variant, was used to isolate transparent (TOT) revertants. The cultures were incubated without agitation at 37° C and periodically sampled. Serial dilutions of transparent cultures were made in BSG, spread on MGE agar and incubated at 37° C for 4 wk to identify O variants. BSG dilutions of opaque cultures were spread on MGE agar containing 25 μg/ml D-cycloserine to inhibit growth of opaque colonies so that T revertants could be counted. Preliminary experiments determined that only T variants grew at concentrations of greater than 15 μg/ml D-cycloserine. These plates were incubated at 37° C for 3 wk and T revertants were identified. New variants were streaked three times for isolation in series on the isolation medium to verify colony type before plasmid analysis.

Plasmid DNA detection. Cultures of mycobacteria were grown at 37° C in MGE to late log-phase (12 d) in 185mm x 16mm screw-cap culture tubes. After bringing the culture to 15% (vol/vol) OA for opaque variants and 20% OA (vol/vol) for transparent variants, the cultures were incubated for an additional 24 to 36 h more at 37° C with rotation (10 RPM on a TC-6 Rollordrum, New Brunswick Scientific, Edison, NJ). D-cycloserine and ampicillin

(Crawford and Bates, 1979) were added to a final concentration of 1.0 and 0.1 mg/ml respectively, and incubation continued for 24 hours at 37° C with rotation. After harvest, cells were lysed by a modification (Jucker and Falkinham, 1990) of the Kado and Liu procedure (1981). This procedure produces crude plasmid DNA that requires further treatment for applications (*e.g.*, restriction digestion and cloning) other than plasmid profile analysis. Plasmid profiles of the Va14 variants were examined by electrophoresis through a 0.5% agarose gel (3 V/cm). The gels were stained with ethidium bromide (0.5 μg/ml) and photographed.

Isolation of total plasmid DNA. Total mycobacterial plasmid DNA was prepared for restriction digestion and subsequent hybridization as follows. Cell lysates prepared as described above were frozen at -70° C until solid. Upon thawing, the DNA solution was centrifuged (14,000 x *g*, 4° C for 5 min) to remove additional phenol and solids and the upper aqueous phase was loaded into prepared dialysis tubing (Sambrook *et al.*, 1989) secured by Spectrapor tubing closures (Fisher Sci. Co., Pittsburgh, PA). The crude DNA solution was dialyzed at 4° C against two changes of at least 100 volumes of dialysis buffer [10 mM Tris-HCl pH 8.0, 5 mM EDTA, and 0.5% (wt/vol) sodium dodecyl sulfate (SDS)] and then treated with 50 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 2 h at 43° C. The DNA solution was cooled to 22° C, vortexed with an equal volume of phenol-chloroform-isoamyl alcohol (PCIA, 25:24:1) and centrifuged (14,000 x *g* for 20 min at 4° C) to separate the phases. The aqueous phase was extracted with chloroform-isoamyl alcohol (CIA, 24:1), centrifuged, and transferred to a clean tube containing 0.3 vol of 7.5 M ammonium acetate. The DNA solution was held in an ice/water slurry (0° C) for 20 min and then centrifuged

(14,000 x g for 20 min at 4° C) to pellet any remaining contaminants (Jucker and Falkinham, 1990). An equal volume of 2-propanol was mixed with the DNA solution and the mixture allowed to stand for 20 min at room temperature to precipitate the DNA. The DNA was pelleted by centrifugation at (14,000 x *g* for 20 min at 4° C), washed with 70% ethanol, and suspended in TE. Restriction endonuclease digestion was carried out following manufacturer's instructions.

Individual plasmid isolation. After initial cell lysis (described above), the individual plasmids of *M. intracellulare* strain Va14 variants were separated by electrophoresis in low-melting-point agarose (Fisher Scientific, Fair Lawn, NJ). Plasmid DNA restriction fragments needed for mapping, cloning, and probe synthesis were prepared by in-gel restriction digestion followed by phenol extraction (Via and Falkinham, 1991b; Chapter 2).

Genomic DNA isolation. Mycobacteria were grown at 37° C with shaking (100 rpm) on an orbital shaker (Labline Instruments, Inc., Melrose Park IL) in 250 ml MGE broth in 500 ml screw-cap Erlenmeyer flasks. After 7 d, OA was added to bring the concentration to 15% (vol/vol) for O variants and 20% (vol/vol) for T variants and incubation was continued for 24 to 36 h. At that time, D-cycloserine and ampicillin were added to final concentrations of 1 mg/ml and 0.1 mg/ml respectively and incubation was continued for an additional 24 h. The cells were harvested (8,000 x *g* for 20 min at 4° C), washed in 0.25 vol TEN [50 mM Tris-Cl pH 8.0, 100 mM EDTA, and 150 mM NaCl, (Patel *et al.*, 1986)] and resuspended in 3 to 6 ml of TEN depending on pellet size. The cells were then treated with 4 mg/ml Subtilisin Carlsberg (protease VIII, Sigma Chemical Co., St. Louis, MO) at 37° C for 3 h. The cells were lysed by incubation at 37° C in 1% (vol/vol) SDS and 3 mg/ml Pronase E (protease

XXVIII, Sigma Chemical Co., St. Louis, MO) for 12 h or overnight (McFadden *et al.*, 1987).

The proteins and nucleic acids were separated by the addition of 0.2 vol of 5 M $NaClO_4$ (Marmur, 1961). The cell lysate was extracted with an equal volume of phenol, 3% (wt/vol) NaCl on a wrist-action shaker (Burrell Corp. Pittsburgh, PA) for 20 min at maximum arc, and centrifuged at 10,000 x g for 20 min at 4° C to separate the phases. All subsequent mixing and extraction steps utilized wrist-action shaking. The aqueous phase was extracted repeatedly with PCIA until no white precipitate was visible at the interface after centrifugation (14,000 x *g* for 20 min at 4° C). The aqueous phase was overlaid with 2 vol of -20° C 95% ethanol and the DNA was spooled onto a glass rod as described by Marmur (1961). After washing the spooled DNA in -20° C 70% ethanol, the DNA was air dried, and dissolved in 2 ml of sterile TE (Sambrook *et al.*, 1989). The DNA solution was treated sequentially with RNase A (Sigma Chemical Co., St. Louis. MO) for 30 min at 37° C and proteinase K for 2 h at 43° C (Sambrook *et al.*, 1989). The protein contaminants were removed from the DNA solution by PCIA extraction as described above, followed by a 10 min CIA extraction with shaking. In a clean tube, 0.3 vol 7.5 M NH_4OAc and 2 vol of -20° 95% ethanol was added to the DNA solution and mixed by gentle inversion. The DNA was spooled, washed as described above, and dissolved in 0.2 to 1 ml TE. Restriction endonuclease digestion was carried out overnight following the enzyme manufacturer's instructions.

Genomic and plasmid DNA hybridization. After electrophoresis, the DNA in agarose gels was depurinated in 0.25 M HCl for 15 min and denatured in 0.4 M NaOH for 30 min before alkaline capillary transfer to Zeta-Probe membranes according to manufacturer's instructions (Bio-Rad Laboratories, Inc., Richmond, CA). DNA probes (3 x 10^5 cpm/ml

hybridization solution) were hybridized with genomic and total plasmid DNA at 50° C in 50% formamide, 4X SSPE (0.72 M NaCl, 40 mM Na_2HPO_4, 1 mM EDTA, pH 7.4), 1% SDS and 0.5% BLOTTO (Sambrook *et al.*, 1989), for 16 h with agitation. The membranes were rinsed in 2X SSC (0.3 M NaCl, 30 mM trisodium citrate pH 7.0; Sambrook *et al.*, 1989) and washed successively with agitation in 1X SSC-0.1% SDS, 0.5X SSC-0.1% SDS, 0.1X SSC-0.1% SDS, and 0.1X SSC-1.0% SDS (20 min each wash) according to manufacturer's directions. Autoradiographs of air-dried membranes were exposed at room temperature using Kodak X-Omat film.

Construction of DNA probes. Mycobacterial DNA fragments were released from recombinant plasmids by restriction endonuclease digestion and separated from the remaining DNA fragments by electrophoresis in SeaPlaque GTG agarose (FMC BioProducts, Rockland, ME). These restriction fragment bands were in-gel radiolabeled with α-^{35}S-dCTP or [α^{32}P]dCTP (1,000 Ci/mmol; New England Nuclear Corp., Cambridge, MA) using a random-primed DNA labeling kit (Boehringer-Mannheim, Inc., Indianapolis, IN).

DNA sequencing. The 1.5 kb *Apa*I-*Sma*I and 0.5 kb *Pvu*II-*Apa*I DNA fragments from pVT328 were ligated into pBluescriptSK (+) and (-) phagemids (Stratagene, La Jolla, CA), and electroporated (GenePulser, Bio-Rad Laboratories, Inc., Richmond, CA) into *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA). Single-strand DNA (ssDNA) was rescued using VCSM13 helper phage following manufacturer's instructions (Stratagene, La Jolla, CA). The ssDNA preparations were sequenced by the dideoxynucleotide chain termination method (Sanger and Coulson, 1975) using the Sequenase 2.0 kit (U.S. Biochemical Corporation, Cleveland, OH) and 50° C termination reactions. The completed reaction products were

separated by electrophoresis through 6% (wt/vol) acrylamide gels containing 40% (vol/vol) formamide. Sequencing was initiated with T7 and T3 primers (U.S. Biochemical Corp.) and new sequencing oligonucleotide primers were synthesized (Oligonucleotide Service Center at VPI&SU) based on newly determined sequence.

Analysis of nucleotide and predicted amino acid sequences. Sequence data were assembled and analyzed using the Sequence Analysis Software Package (Genetics Computer Group Inc., Madison, WI; Devereux *et al.*, 1984) through a VPI&SU VAX computer. Searches of GenBank and EMBL data bases (Bilofsky and Burks, 1988) were carried out using Wordsearch (Wilbur and Lipman, 1983) and Tfasta (Pearson and Lipman, 1988). Codonpreference (Gribskov *et al.*, 1984) and Testcode (Fickett, 1982) programs provided with a table of preferred codon usage for mycobacteriophage L5 (Hatfull and Sarkkis, 1993) were used to identify potentially translated open reading frames (ORFs). Multiple amino-acid sequence alignments were prepared with GCG Pileup (Devereux *et al.*, 1984) with manual consensus adjustment.

Nucleotide sequence accession number. The sequence data reported here have been submitted to GenBank and assigned accession number L10239.

Determination of IS*1141* copy number. Using the primers LI (5'GTCAACCTGAATGAGGCACTGTTG) and 660(+) (5' CACGCCCACAGCTCTCCGACGAC), a [α^{32}P]dCTP labeled, 380 bp DNA fragment was synthesized by polymerase chain reaction to hybridize with the right end of IS*1141*. Primers RI (5' TGCCAACCTGGGTTGAGGCACCTG) and 1261(-) (5' CGGCATCGCAGCGTTGCGTAC) were used to produce a similarly labeled 600 bp left-end

probe. Synthesis of probes specific to the left end and right end of the IS element was carried out using *Taq* DNA polymerase (Promega Corp, Madison, WI) in a standard reaction (Sambrook *et al.*, 1989) in a PTC-60 thermocycler (MJ Research, Watertown, MA). Each probe was hybridized with a separate, identically prepared Southern blot containing the *Eco*RV digested Va14 colonial-variant DNAs as described above.

Measurement of rate of variation. To determine the rate of colonial variation in strain Va14, single colonies of either Va14(O) or Va14(T) were suspended in 2 ml MGE broth and incubated for 4 d at 37° C. These stock cultures were used to inoculate duplicate 7 ml cultures of MGE medium in optically matched 150mm x 16mm screw-cap culture tubes. The 7 ml cultures were incubated with rotation (10 RPM on a TC-6 Rollerdrum, New Brunswick Scientific Co., Edison, NJ) at 37° C. The cultures were sampled immediately and every 2 d throughout log-phase growth (10 d). Growth was monitored as increase in absorbance at 580 nm with a spectrophotometer (Junior, Coleman Spectrophotometer Corp., Maywood, IL). The wavelength was chosen to eliminate absorbance by any yellow carotenoid pigments (Stormer and Falkinham, 1989). Cultures of Va14(T) were serially diluted in BSG and plated on MGE agar. Numbers of O and T colonies were counted after incubation at 37° C for 3 wk. Cultures of Va14(O) were diluted in BSG and plated on MGE agar to determine the number of opaque CFU/ml and on MGE agar containing 25 μg/ml D-cycloserine to select for growth of T colonies. After incubation for 3 wk at 37° C, the colonies were counted. Rates of colonial variation were calculated by a modification of the Witkin method (Witkin, 1950).

Growth profiles of variants. The growth of transparent and opaque colonial variants was measured in M7H9 medium containing 10% ADC (BBL Microbiology Systems,

Cockeysville, MD) alone or with 0.5% (vol/vol) Tween 80 (Kajioka and Hui, 1978). Cultures were incubated at 37° C with rotation (10 RPM) in optically matched, 150mm x 16mm screw-capped tubes. Growth of T and O cultures was reflected by increases in absorbance measured at 580 nm.

Antibiotic susceptibility of variants. The antibiotic susceptibility of *M. intracellulare* Va14 transparent and O variants was compared by determining the broth microdilution MICs using the Sceptor system panels (BBL, Microbiology Systems, Cockeysville, MD) as described by Yajko *et al.* (1987).

RESULTS

Plasmid profiles. The plasmid DNA of opaque and transparent colonial variants of *M. intracellulare* strain Va14 was separated by agarose gel electrophoresis (Fig. 3.1). The plasmid profile (240, 68, 25, and 16 kb) observed in O strain LV1 was shared by 42 of 42 sibling colonies examined (Fig. 3.1A, lane 2). Two profiles have been seen in transparent derivatives of strain LV1, the 240, 62, 25, and 16 kb profile was shared by strains LV11 and LV15 (lane 3) and the parental (240, 68, 25, and 16 kb) profile shared by variant LV12 and 13 other independent derivatives (lane 4). The plasmid profile (240, 62, 25, and 16 kb) of the parental transparent strain LV2 was shared by 38 of 38 sibling colonies examined (Fig. 3.1B, lane 2). Opaque derivatives of strain LV2 (T) were found to have one of three profiles; the 240, 68, 25, and 16 kb profile shared by variants LV21, LV219 and nine other independent derivatives (lane 3), the 240, 68, 25, and 18 kb profile of variant LV26 (lane 4), and the parental (240, 62, 25, and 16 kb) profile was seen in two derivatives (lane 5). The seven OTO and the eight TOT derivatives examined had the 240, 68, 25, and 16 kb plasmid profile (Fig 3.1B, lane 3). Throughout eight years of culture only the 68 and 16 kb plasmids have been observed to change size. A change from 16 to 18 kb has been observed in two separate variants (one of which formed rough colonies) while the change from 62 to 68 kb has been observed multiple times (Table 3.1).

Restriction endonuclease mapping and cloning of the unique sequence from pVT203. The opaque strain LV1 was found to contain a 68 kb plasmid (pVT203), while the transparent derivative LV11 contained a 62 kb plasmid (pVT204) similar in size to the plasmid

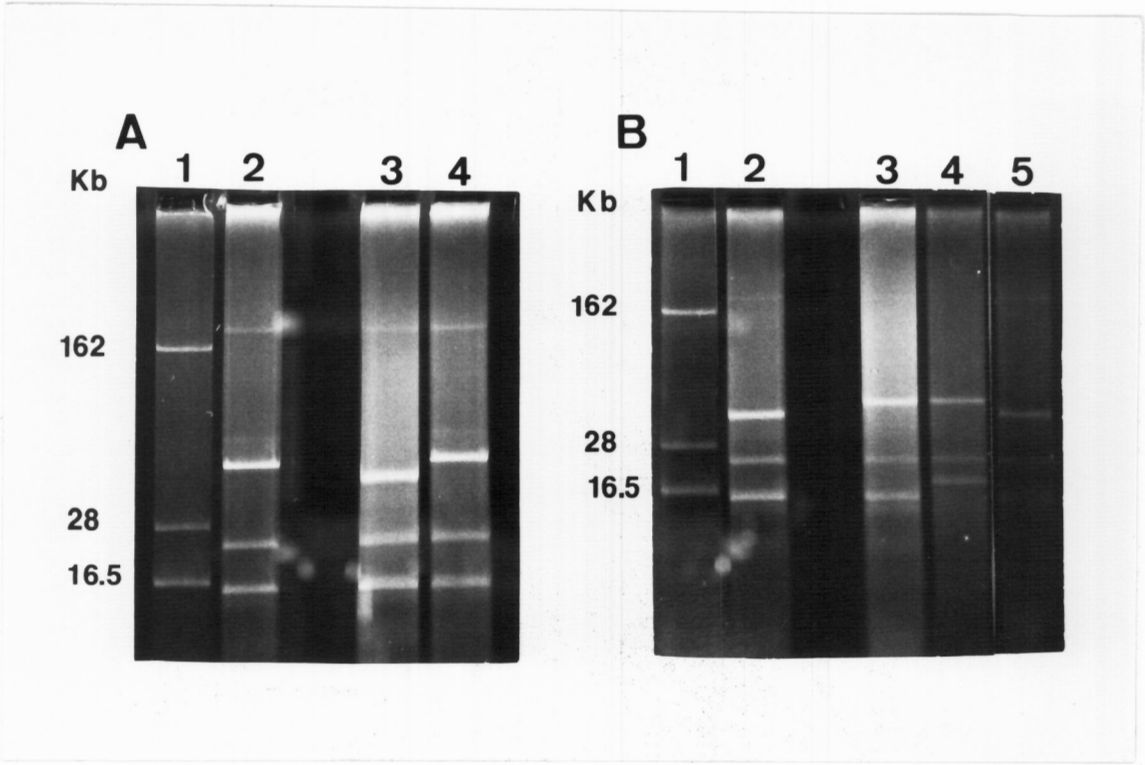

FIG. 3.1. Plasmid profiles of *M. intracellulare* strain Va14 opaque (O) and transparent (T) colonial variants. In both A and B, lane 1 contains plasmids (162, 28, 16.5 kb) from *M. avium* strain LR25 as molecular mass markers (Crawford and Bates, 1979). Panel A shows plasmids of LV1 (O) (lane 2) and the plasmid profiles of its T derivatives (lanes 3 and 4). Panel B shows plasmids of LV2 (T; lane 2) and the plasmid profiles of O derivatives (lanes 3, 4, and 5).

observed in the transparent parent LV2. Restriction digests of the plasmids pVT203 and pVT204 with *Hin*dIII and *Eco*RI followed by gel electrophoresis resulted in only single band differences (data not shown). In *Hin*dIII digests of pVT203, a 18 kb band was present rather than the 12 kb band found in pVT204 *Hin*dIII digests; in *Eco*RI digests of pVT203, a 12 kb band was present rather than the 6 kb band found in pVT204 *Eco*RI digests. No difference in *Hin*dIII or *Eco*RI restriction digests of the 62 kb plasmids from LV2 and LV11 were observed (data not shown). Figure 3.2 illustrates restriction endonuclease maps of pVT203 and pVT204. DNA restriction fragments from *Hin*dIII-*Eco*RI double digests of the two plasmids were ligated into pBR322 or pUC19 vectors and transformed by electroporation (GenePulser, Bio-Rad Laboratories, Inc., Richmond, CA) into *E. coli* strain DH5α (Hanahan, 1983). Recombinant plasmids containing the 11.3 kb *Hin*dIII-*Eco*RI fragment of pVT203 (pVT328) and the corresponding 6 kb *Hin*dIII-*Eco*RI fragment of pVT204 (pVT408) were identified by hybridization of DNA from lysed *E. coli* colonies on nylon membranes (Sambrook *et al.*, 1989) with the radiolabeled 18 kb *Hin*dIII restriction fragment of pVT203 (Fig. 3.2) and restriction endonuclease maps were constructed (Fig. 3.3).

From inspection of pVT328 and pVT408 restriction maps, we identified a 3.3 kb region between the interior *Sal*I and *Bam*HI restriction sites that appeared to be unique to pVT328 (Fig. 3.3) and thus might contain the putative transposable element. Flanking either side of the 3.3 kb unique region, there appeared to be a duplication of approximately 1.8 kb of DNA (*Bam*HI-*Pvu*II region) which was not duplicated in pVT408 (*i.e.*, the 1.8 kb region was present only once; Fig. 3.3). Hybridization of restriction endonuclease digested pVT328 and pVT408 plasmids with any one of these three 1.8 kb fragments showed that each fragment

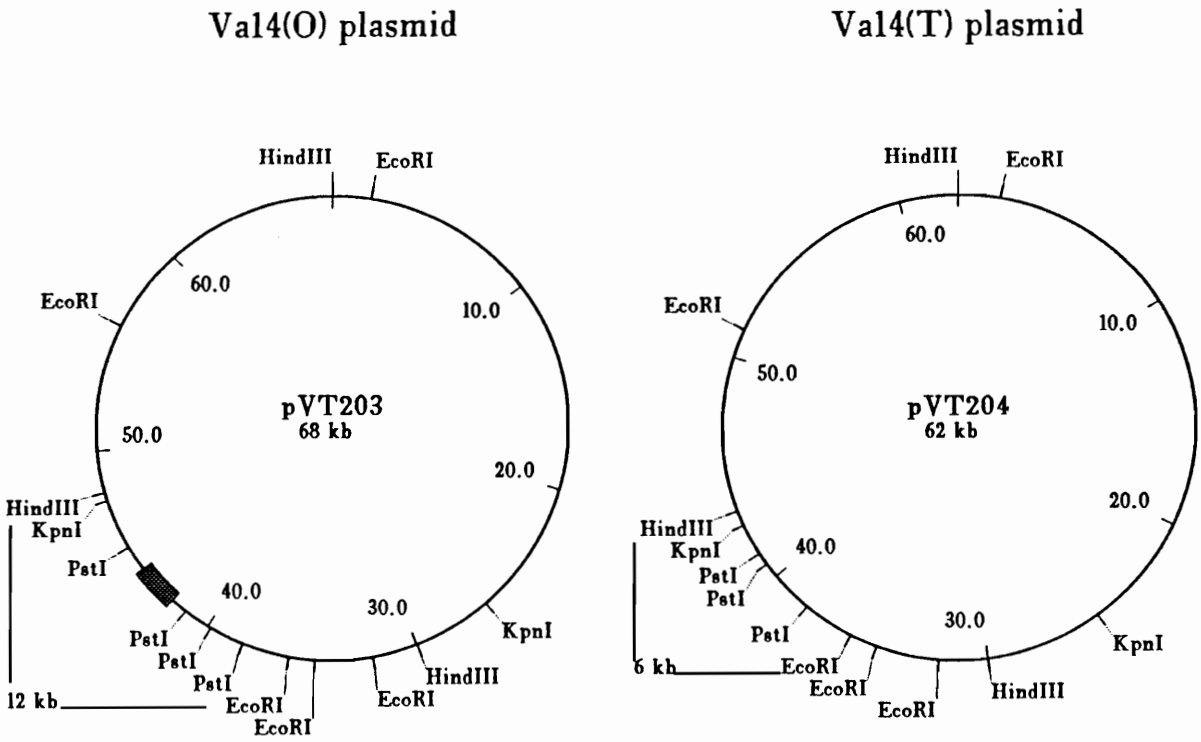

FIG. 3.2. Restriction maps of *M. intracellulare* strain Val4 pVT203 and pVT204 plasmids. The plasmids contain three *Hin*dIII sites; only selected sites for the other endonucleases (*Eco*RI, *Kpn*I and *Pst*I) are shown. The marked 12 kb *Hin*dIII-*Eco*RI fragment of pVT203 was cloned into pBR322 creating pVT328 and the corresponding 6 kb fragment from pVT204 was cloned into pUC19 producing pVT408 (Fig. 3.3).

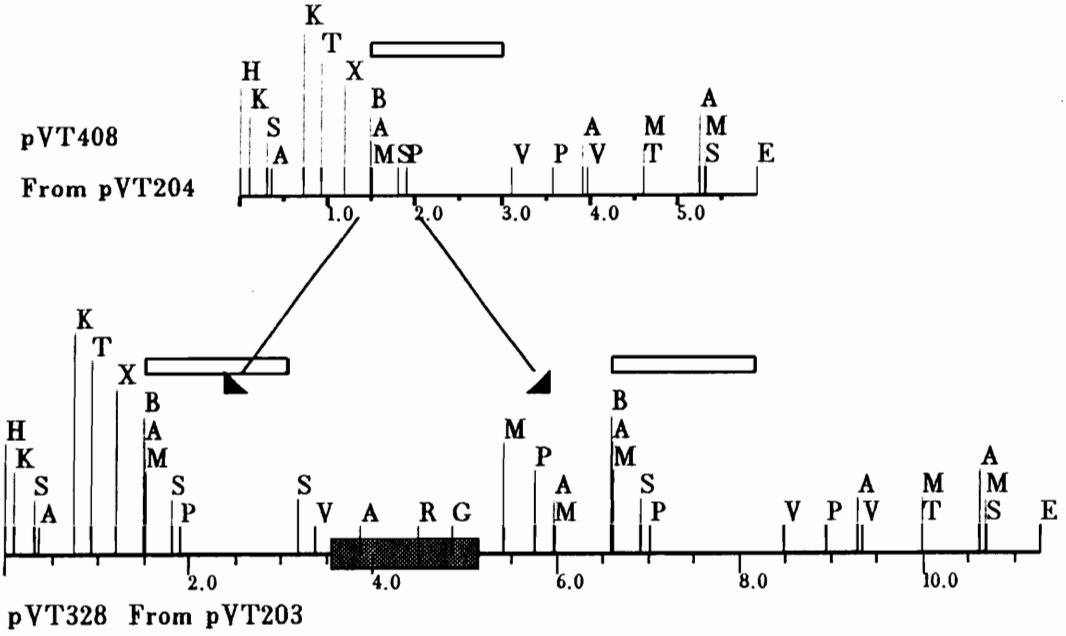

FIG. 3.3. Restriction maps of *M. intracellulare* strain Va14 plasmid DNA *Hin*dIII-*Eco*RI restriction fragments cloned into pBluescript(+/-) phagemids as pVT408 (5.8 kb insert) and pVT328 (11.2 kb insert). Arrows mark the areas where the restriction maps differ. Shaded block marks position of IS element. Open blocks indicate regions in pVT408 which appear duplicated in pV328. The symbols used in the map refer to the following restriction endonucleases: A *Apa*I, B *Bam*HI, E *Eco*RI, G *Bgl*II, H *Hin*dIII, K *Kpn*I, M *Sma*I, P *Pst*I, R *Eco*RV, S *Sal*I, T *Sst*I, V *Pvu*II and X *Xho*I.

hybridized to the other two 1.8 kb regions (data not shown). Results from additional hybridization experiments suggested that the duplicated region in pVT328 extended upstream of the *Bam*HI site toward the *Sma*I site (Fig. 3.3).

To confirm the uniqueness of the 3.3 kb *Sal*I-*Bam*HI fragment of pVT328, the fragment was labeled with α^{35}S-dCTP and used to probe a Southern blot of plasmid DNAs from strain LV1 [pVT203, opaque] and variants LV11 [pVT204, transparent], LV2 (transparent) and LV26 (opaque). The probe hybridized with the 68 kb plasmids of variants LV1 and LV26 as expected, but it also hybridized with the 16 kb (strain LV1 (pVT206) and variants LV11 and LV2) and 18 kb (variant LV26, pVT205) plasmids (Fig. 3.4). These hybridization results suggested that the 3.3. kb *Sal*I-*Bam*HI fragment from the 68 kb plasmid was absent from the 62 kb plasmid (variants LV11 and LV2), but that the fragment was present elsewhere in the plasmids (lanes 1-4). Hybridization of the 3.3 kb probe with restriction endonuclease fragments of the 18 kb and 16 kb plasmids and the chromosome showed that some hybridizing bands were smaller than 3.3 kb (data not shown). Additional hybridization analysis of pVT328, pVT206, and the chromosome indicated that the 0.5 *Pvu*II-*Apa*I and 1.5 kb *Apa*I-*Sma*I restriction fragments of pVT328 were the parts of the 3.3 kb unique region present in multiple copies in strain Va14 and thus likely to contain the putative transposable element.

Hybridization of putative transposable element with genomic DNA. To determine if the unique fragment from pVT203 was present in the chromosome of Va14 strains, the α^{35}S-dCTP-labeled 1.5 kb *Apa*I-*Sma*I fragment from pVT328 (see Fig. 3.3) was hybridized with Southern blots of genomic and total plasmid DNA restriction digests. The restriction

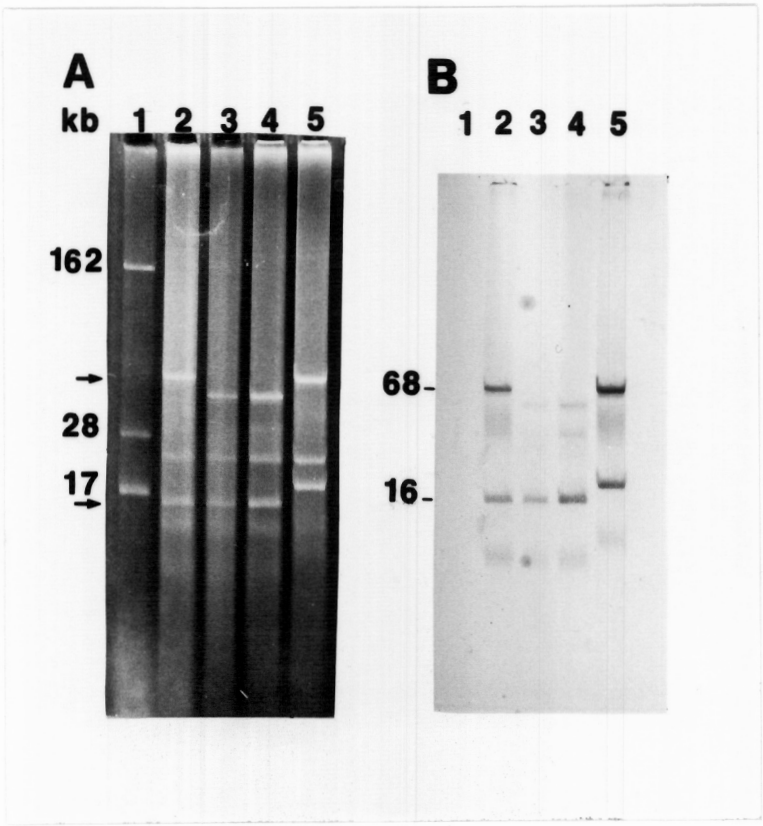

FIG. 3.4. *M. intracellulare* strain Va14 opaque (O) and transparent (T) colonial variant plasmid DNA profiles (A) and Southern hybridization (B). Plasmids from *M. intracellulare* Va14 LV1(O) [lane 2], LV11(OT) [lane 3], LV2(T) [lane 4], and LV26(TO) [lane 5] were hybridized to the 3.3 kb *Bam*HI-*Sal*I restriction fragment from pVT203 (Fig. 3.3) containing the IS element. Lane 1 contains plasmids from *M. avium* strain LR25 as molecular mass standards.

endonucleases used for these experiments did not cleave the IS element. Since the genomic DNA samples contained both the bacterial chromosome and the plasmids, total plasmid digests were included to distinguish hybridizing bands that originated from the plasmids and those from the chromosome. For each restriction endonuclease listed in Fig. 3.5, genomic DNA samples from strain LV2 (T), strain LV1 (O), and plasmid (P) DNA from strain LV1 were digested and separated by electrophoresis. The transparent strain LV2 appears to have two chromosomal bands which hybridize with the element probe and the opaque strain LV1 shows two or three chromosomal bands which hybridize to the probe. For all six restriction endonucleases, one of the hybridizing chromosomal bands appeared to be the same size in O and T strains, while the other hybridizing chromosomal bands differed in size among the O and T variants.

Nucleotide sequence of the transposable element. The strategies used to sequence the 0.5 kb *Pvu*II-*Apa*I (pVT361) and 1.5 kb *Apa*I-*Sma*I (pVT354) subclones from pVT328 are shown in Fig. 3.6. Concurrently, the 370 bp *Eco*RV-*Bgl*II fragment was subcloned into pBluescriptSK and sequenced with T7 and T3 primers. In order to sequence across the *Apa*I site, the 2.4 kb *Sal*I-*Sma*I fragment from pVT328 was subcloned (pVT365). In this manner, overlapping sequences were generated.

Since no definite terminal inverted repeats were found initially, fragments from the 16 kb plasmid (pVT450) and the chromosome (pVT460) that hybridized with the 1.5 kb *Apa*I-*Sma*I fragment were subcloned and partially sequenced using the primers whose names are shown in Fig. 3.6. The nucleotide sequence of the putative transposable element was identified by comparison of the sequenced portions of the subclones pVT450 and pVT460 with

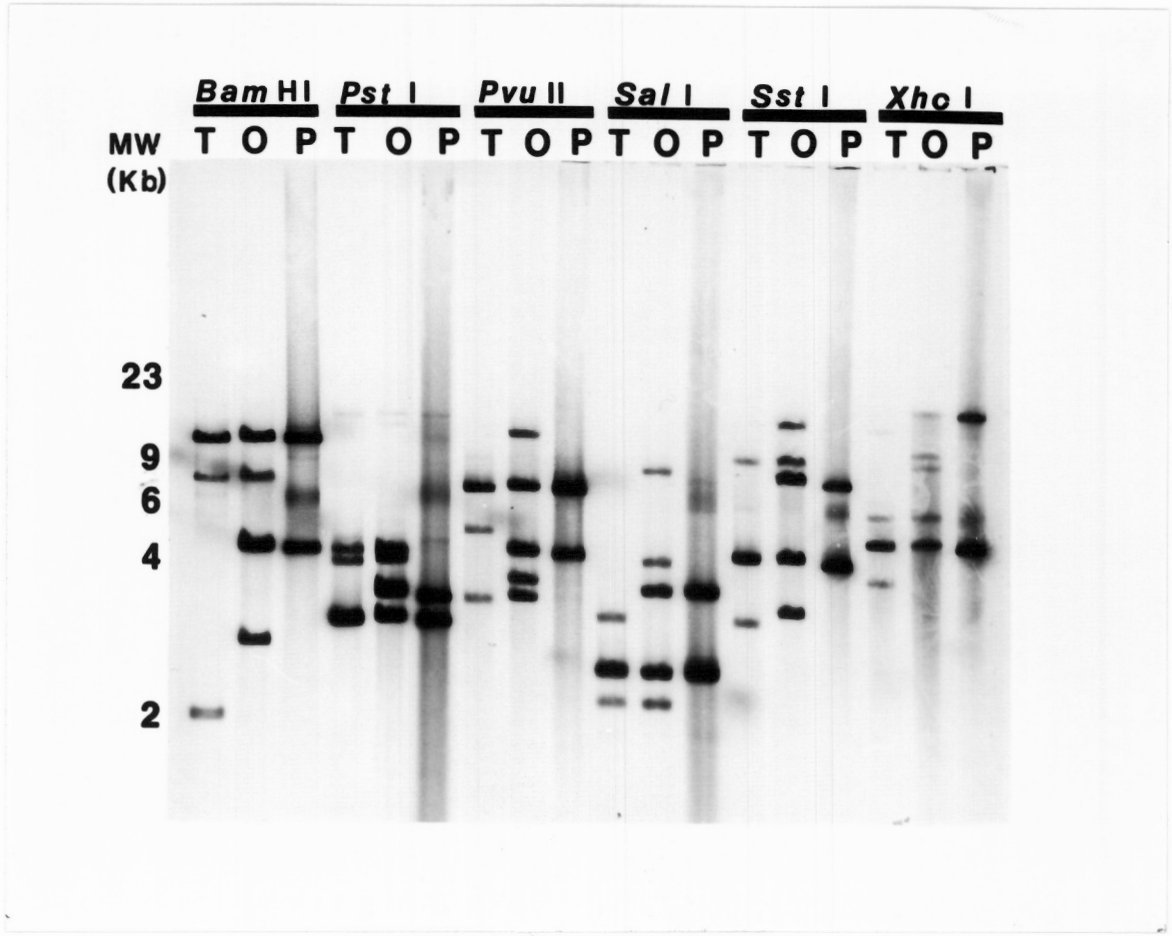

FIG. 3.5. RFLP analysis of *M. intracellulare* strain Va14 opaque (O) and transparent (T) colonial variants. Hybridization of IS*1141* probe (1.5 kb *Apa*I-*Sma*I fragment) with restriction endonuclease-digested plasmid and genomic DNA from *M. intracellulare* strains LV2 (T) and LV1 (O). Three DNA samples were cleaved: LV2 (T), LV1 (O), and plasmid DNA (P) from LV1 with each enzyme. The molecular mass markers (kb) to the left of the figure indicate the position of *Hin*dIII-digested lambda DNA.

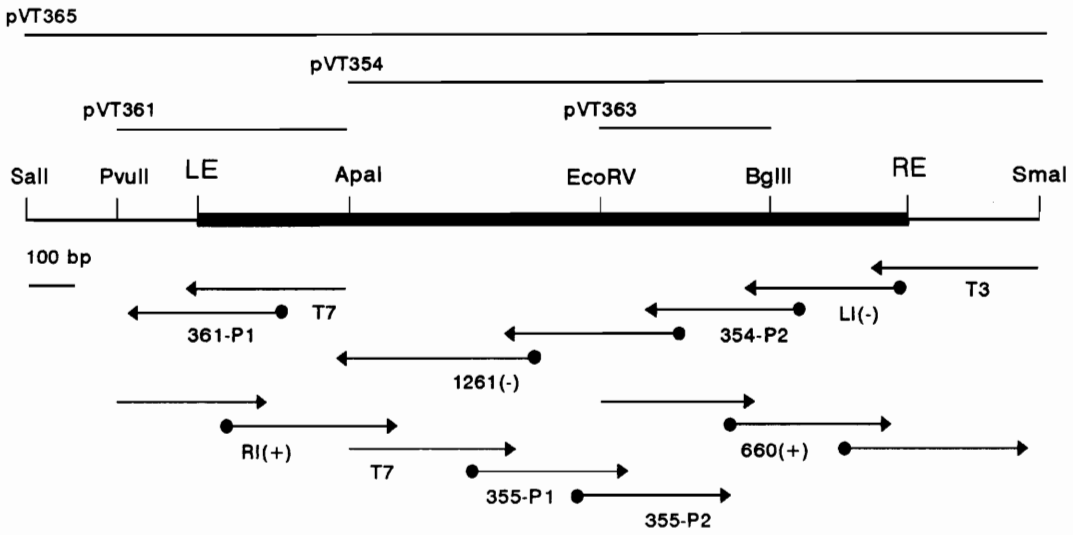

FIG. 3.6. Recombinant plasmids constructed and sequencing strategy. The lines above the restriction map show the subclones of pVT328 (Fig. 3.3) constructed for sequencing. The arrows below the map show the sequencing strategy. Some primers (arrows with solid circle anchor) were synthesized to sequence areas that could not be traversed with the subclones and T7 and T3 primers for pBluescript (-/+). The named primers were used to sequence both the fragments of pVT203 and the fragments from the 16 kb plasmid and the chromosome except primers RI(+), 1261(-) and 660(+). Primers RI(+), LI(-), 1261(-) and 660(+) were also used in PCR probe production.

the sequence from the subclones (pVT354 and pVT361) of 68 kb plasmid (Fig. 3.7). The element was determined to be 1596 nucleotides (nt) in length with a G + C content of 66% and to have imperfect (18 of 23 nt complementary) inverted repeats located at positions 6 to 28 and 1570 to 1593 (Fig. 3.8). On the basis of the hybridization data, the sequence data above, and the ORFb putative amino-acid sequence similarity to other IS elements below, the 1596 bp sequence was designated as insertion sequence IS*1141*

Analysis of potential coding regions. Seven open reading frames greater than 50 amino acids in length were identified (Figure 3.9). Three of these on the plus strand, ORFa (nt 184 to 534), ORFb (nt 536 to 1559), and ORFc (nt 63 to 538) and two on the reverse stand, ORFf1 (nt 1194 to 1016) and ORFf2 (nt 786 to 620) were indicated as potentially translated regions by measuring the non-randomness of nucleotide composition in the third position of each reading frame (GCG; Fickett *et al.*, 1982) and by codon bias analysis (GCG; Gribskov *et al.*, 1984). The best available Shine-Dalgarno (SD) sequences for ORFs a, b, and c are shown (Fig. 3.8). ORFa would code for a 15 kDa protein with a pI of 12.8. ORFc has several potential start codons, but positional base-preference and codon bias analysis suggested that translation begins beyond the initial ATG (base 63). The best available SD sequence in ORFc precedes the ATG at position 138. If this translation start is used, a 17 kDa protein with a pI of 10.2 would be produced. The sequence proceeding the ORFb start codon (GTG, base 536) has little resemblance to the consensus SD sequence, but both ORFc and ORFa have termination codons within the region of the ORFb start codon, so ribosomal re-initiation may be possible for translation of ORFb. The putative protein product of ORFb is a 43 kDa basic protein (pI 10).

Origin (clone)	5′ element-junction	3′ element-junction
pVT203 (pVT365)	**GCTG**CCCCCCA***CGTAA***▌CCCTCTCTG.....GACAGATTC▌***CGTAA***CCCACTGTCC	
pVT206 (pVT450)	**GCTG**TCAAGGC***TCGAC***▌CCCCTCTCTG....GACAGATTC▌CGCCTGG***TCGAC***GCG	
6 kb *Eco*RI (pVT460)	**GCTG**ATCGGTCAGC***GCGA***▌CCCTCTCTG....GACAGATTC▌***GCGA***CCCTGTGGTGT	

IS*1141* Consensus insertion site **GCTG**$N_{(2-6)}$CA(N)GNNNNNNNCCTG(G)T
▲

IS*900* Consensus insertion site **G**NCATGNNNNNNNCCT
▲

FIG. 3.7. Sequence comparison of insertion sites of three copies of IS*1141*. Nucleotides that are conserved at the three insertion sites are underlined or marked in bold. Those conserved insertion site sequences similar to the sequences seen in IS*900* are underlined. The suggested target duplication for the three IS*1141* copies is shown in bold italics. The consensus target insertion site for IS*1141* and IS*900* (Kunze *et al.*, 1991) are shown for comparison.

```
      IRL ------------------------->
  1   CCCTCTCTGC CAACCTGGGT TGAGGCACCT GCCCCCTGAC AATTAGTGAC   50
      GGGAGAGACG GTTGGACCCA ACTCCGTGGA CGGGGGACTG TTAATCACTG
            -35 region                        -10 region

        --SD-      ORFc->
  51  CCGAGGGCGG GGATGGAGAG CTTCCCCAAG ATGGCGAACA CGGTGACCAC   100
      GGCTCCCGCC CCTACCTCTC GAAGGGGTTC TACCGCTTGT GCCACTGGTG
                  -35 region
                                --SD-
 101  ATTGGTCCGA TCGACCGGGA TCGAAGATCC TGGCCGGATG AACGACGCTG   150
      TAACCAGGCT AGCTGGCCCT AGCTTCTAGG ACCGGCCTAC TTGCTGCGAC

                           ----SD--        ORFa->
 151  GGACGGAACG TCCCGACCCT GAGGTGCCCG AGCGTGCCCG ACGCCGGACG   200
      CCTGCCTTGC AGGGCTGGGA CTCCACGGGC TCGCACGGGC TGCGGCCTGC

 201  TTCACCGCGA AGTACAAGCT GGAGATCCTG GCCGCTTACG ACGCTGCTCC   250
      AAGTGGCGCT TCATGTTCGA CCTCTAGGAC CGGCGAATGC TGCGACGAGG

 251  CGAAGGCGAG AAGGGTGCGC TGTTGCGCCG GGAGGGGCTG TATTCCAGCC   300
      GCTTCCGCTC TTCCCACGCG ACAACGCGGC CCTCCCCGAC ATAAGGTCGG

                           ApaI
 301  ACATTGTGGC GTGGCGGCGG GCCCGCGACG CCGGCGCGTT GGCTGGCTTG   350
      TGTAACACCG CACCGCCGCC CGGGCGCTGC GGCCGCGCAA CCGACCGAAC

 351  GCCGTTCCGC GCGGACGTAA GCGGCGCGAC CCACAGGGCG AGCGGATCGC   400
      CGGCAAGGCG CGCCTGCATT CGCCGCGCTG GGTGTCCCGC TCGCCTAGCG

 401  CCGGCTGGAG GCCGAAAAAC ACCAACTGGA GCAGGAGCTG GCCAAGACCC   450
      GGCCGACCTC CGGCTTTTTG TGGTTGACCT CGTCCTCGAC CGGTTCTGGG

 451  GCTTCGTGGT GGACGTCCAG GCAAAACTGC ACGCGCTCTT GGAGACGCTC   500
      CGAAGCACCA CCTGCAGGTC CGTTTTGACG TGCGCGAGAA CCTCTGCGAG

                           --SD--          ORFb->
 501  TCCGAGAGCG CGGAGCCCGA GAACGGGTCG ATGAAGTGAG CGACGCAGCG   550
      AGGCTCTCGC GCCTCGGGCT CTTGCCCAGC TACTTCACTC GCTGCGTCGC

 551  ATCAGCGAGC TGGCGCCCAA GATCGGCGTA CGCAACGCCT GCGATGCCGT   600
      TAGTCGCTCG ACCGCGGGTT CTAGCCGCAT GCGTTGCGGA CGCTACGGCA

 601  CGGTGTCGCC CAAGCCAGCT ACTACCGCCG GCACCGCAAA GCCCGCCACC   650
      GCCACAGCGG GTTCGGTCGA TGATGGCGGC CGTGGCGTTT CGGGCGGTGG

 651  GCAGCGGCCG GCGCCGATCC CGCACACCGA CCGGGTGCAG CCGCGTGCAT   700
      CGTCGCCGGC CGCGGCTAGG GCGTGTGGCT GGCCCACGTC GGCGCACGTA
```

```
 701  TGTCCCGCCG CCGAGCGGGC CGCGATCCTC AATGAGTTGC ACAGCGAGCG   750
      ACAGGGCGGC GGCTCGCCCG GCGCTAGGAG TTACTCAACG TGTCGCTCGC

 751  GTTCATCGAC ACCTCGCCGA CCGAAGTGTG GGCCACACTG CTCGACGAAG   800
      CAAGTAGCTG TGGAGCGGCT GGCTTCACAC CCGGTGTGAC GAGCTGCTTC
                                       <-ORFf2

 801  GCCGCTACCT CGGCTCGATC TCGACCTTCT ACCGGCTGCT GCGCCAAGCC   850
      CGGCGATGGA GCCGAGCTAG AGCTGGAAGA TGGCCGACGA CGCGGTTCGG

 851  GGCGAAAGCC GGGAGCGCCG CCGGCAGGCC ACCCACCCAG CAACGGTAAA   900
      CCGCTTTCGG CCCTCGCGGC GGCCGTCCGG TGGGTGGGTC GTTGCCATTT

                                                    EcoRV
 901  ACCCGAGCTG GTCGCGTTCG AGCCGAACCA GGTGTGGAGT TGGGATATCA   950
      TGGGCTCGAC CAGCGCAAGC TCGGCTTGGT CCACACCTCA ACCCTATAGT

 951  CCAAGCTGCG CGGCCCGGCG AAGTGGAGCT GGTACTACCT CTACGTGATC  1000
      GGTTCGACGC GCCGGGCCGC TTCACCTCGA CCATGATGGA GATGCACTAG

1001  TTGGACATCT TCTCCCGCTA CGTGGTCGGA TGGATGGTCG CTAGTCGCGA  1050
      AACCTGTAGA AGAGGGCGAT GCACCAGCCT ACCTACCAGC GATCAGCGCT

1051  AAGTGCCGCG CTCGCTGAGG TATTGATCCG CCAGACCTGC GCCAAGCAGG  1100
      TTCACGGCGC GAGCGACTCC ATAACTAGGC GGTCTGGACG CGGTTCGTCC

1101  ACATCGGGCG CGACCGGTTG ACCATCCACG CCGACCGCGG CTCCTCGATG  1150
      TGTAGCCCGC GCTGGCCAAC TGGTAGGTGC GGCTGGCGCC GAGGAGCTAC

1151  ACCTCCAAGC CGGTGGCGTT CCTGCTCGCC GACCTCGGCG TCACCCAGTC  1200
      TGGAGGTTCG GCCACCGCAA GGACGAGCGG CTGGAGCCGC AGTGGGTCAG
                                                  <-ORFf1

1201  GCACTCACGC CCACACGTCT CCGACGACAA CCCGTTCAGC GAGGCGCAAT  1250
      CGTGAGTGCG GGTGTGCAGA GGCTGCTGTT GGGCAAGTCG CTCCGCGTTA

1251  TCAAGACGTT GAAATATCGG CCCGACTTCC CCGACCGGTT CGACTCGATC  1300
      AGTTCTGCAA CTTTATAGCC GGGCTGAAGG GGCTGGCCAA GCTGAGCTAG

                              BglII
1301  GAGGCCGCCC GCCGGCACTG CCAGATCTTC TTCGGCTGGT ACAACGACGA  1350
      CTCCGGCGGG CGGCCGTGAC GGTCTAGAAG AAGCCGACCA TGTTGCTGCT

1351  ACATCGCCAT ACCGGGCTGG GTTTACACGT TCCTGCCGAC GTGCACTACG  1400
      TGTAGCGGTA TGGCCCGACC CAAATGTGCA AGGACGGCTG CACGTGATGC
```

```
1401  GCACCGCCGC GATCATCCGC GACAAGCGCG CCGGCGTGCT CGACGCCGCC   1450
      CGTGGCGGCG CTAGTAGGCG CTGTTCGCGC GGCCGCACGA GCTGCGGCGG

1451  TACGCCGCAC ACCCAGAACG GTTCGTGCAA AAGCCGCCCG AACCACCGAA   1500
      ATGCGGCGTG TGGGTCTTGC CAAGCACGTT TTCGGCGGGC TTGGTGGCTT

                                      -35 region
1501  ACTGCCCAGC GGCTCATGGA TCAACAAACC AGACGACACC GAGGAGGCCA   1550
      TGACGGGTCG CCGAGTACCT AGTTGTTTGG TCTGCTGTGG CTCCTCCGGT

        -10 region                      -35 region
1551  TTCAGTAAAT ACCCTCAACA GTGCCTCATT CAGGGTTGAC AGATTC  1596
      AAGTCATTTA TGGGAGTTGT CACGGAGTAA GTCCCAACTG TCTAAG
                         IR_R ------------------------>
```

FIG 3.8. Nucleotide sequence of IS*1141*. The sequence of IS*1141* copies found in pVT203, pVT206 (16 kb plasmid) and a in chromosomal 6.0 kb *Eco*RI-fragment of *M. intracellulare* strain Va14(O). The left (IR^L) and right (IR^R) inverted repeats, the start codons for probable translated ORFs and their possible ribosome-binding sites (S-D) are indicated. The promoter-like sequences at the ends of the element and the major restriction endonuclease sites are labeled.

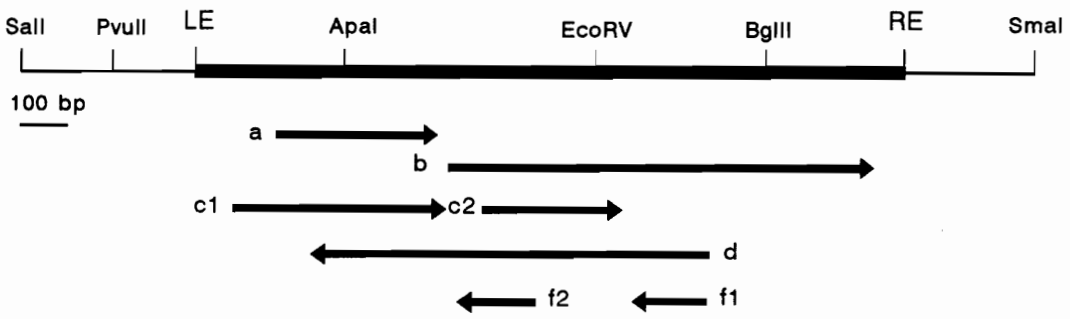

FIG 3.9. Open reading frames (ORFs) of IS*1141*. Each ORF over 50 amino acids in length was named based on it's reading frame. The terminating codons of ORFa and ORFc are within three bases of the GTG of ORFb.

When the GenEMBL data bank (release 74; Bilofsky and Burks, 1988) was searched with the putative amino acid sequence of ORFb (Fig. 3.8 and 3.9) using the GCG Tfasta process (Pearson and Lipman, 1988), ORFb of IS*911* (Prere *et al.*, 1990; accession number X17613) and ORFb of IS*3* (Timmermann and Tu, 1985; accession number X02311) were identified as significantly similar to ORFb. IS*1141* OFRb shares 49% similarity and 29% identity with IS*911* OFRb and 48% similarity and 24% identity with IS*3* ORFb. The search with ORFb also detected significant similarity (48% similarity and 28% identity) with IS elements IS*904* (Rauch *et al.*, 1990; accession number X522730) and IS*981* (Polzin and McKay, 1991; accession number M33933) from *Lactococcus lactis*. Searches with the other open reading frames of IS*1141* did not identify any significant similarity.

To further measure the significance of the similarities observed, the method of Barton (1990) was used. Pairwise alignments of IS*1141* ORFb and the ORFbs of the IS elements above were constructed with the GCG Bestfit program. As the alignments were constructed, the program also aligned randomized amino-acid sequences from IS*1141* ORFb with the original sequence of ORFb for the other elements. The mean and standard deviation of quality scores for the randomized sequence of IS*1141* ORFb were compared with the quality score of each alignment of the actual ORFb and the other IS ORFbs (Barton, 1990). The means of the random-sequence quality scores were 7.5 to 10 standard deviations below the quality scores for the actual alignments of ORFb with the IS*3* family ORFs suggesting that ORFb of the sequence described was significantly similar to the transposases of the IS*3* family.

Analysis using direct nucleotide and predicted amino-acid sequence searches (GCG Wordsearch process; Wilber and Lipman, 1983) did not reveal significant similarity to the

other MAC IS elements IS*900,* IS*901*, and IS*902* (Green *et al.*, 1989; Kunze *et al.*, 1991; Moss *et al.*, 1992). Although data bank searches did not result in identification of IS*986* (IS*6110)* from *M. tuberculosis* as similar to IS*1141*, a pairwise alignment of amino-acid sequences indicated that IS*1141* ORFb and IS*986* ORFb shared significant similarity (48%) and identity (29%). Specific comparison of IS*1141* ORFb with the putative transposases from IS*900* (Green *et al.*, 1989) and IS*901* (Kunze *et al.*, 1991) revealed 17% and 19% identity respectively, but the mean differences in quality scores for the actual and random amino acids sequences were insignificant (Barton, 1990).

A multiple alignment of the putative transposases of IS*1141*, IS*986* (McAdam *et al.*, 1990), IS*911* from *Shigella dysenteriae* (Prere *et al.*, 1990), and IS*3* from *E. coli* strain C600 (Timmermann and Tu, 1985) is presented in Fig. 3.10. Although several conserved regions exist among the four sequences, the IS*1141* ORFb is 40 amino acids longer than all the identified transposase ORF's of the others (Prere *et al.*, 1990). There is a 100 bp region in the transposase coding region with 60% nucleotide identity among IS*1141*, IS*3* and IS*911*. In the alignment, IS*986* and IS*1141* shared many of the IS*3* family regions, but pairwise comparisons reveal no greater similarity between IS*1141* and IS*986* than between IS*1141* and IS*911*.

Junction sites in the genome. IS*1141* shares the presence of imperfect terminal inverted repeats with IS*986* and the IS*3* family elements. These repeats range from 25 to 40 bp in length with as few as 22 perfect matches (Prere *et al.*, 1990). There is 40% sequence similarity between the IS*911* inverted repeats and those of IS*1141*, but the repeats of IS*1141* are recessed from the ends of the element (Fig. 3.8). IS*1141* appears to share target site and terminal sequence similarity (Fig. 3.7) with IS*900* and IS*901*, elements which lack inverted

```
          1                                                  50
IS1141    ....VSDAAI SELAPKIGVR NACDAVGVAQ ASYYRRHRKA RHRSGRRRSR
IS986     .......... .......... .....VPIAP STYYDHINRE PSRRELRDGE
IS3       ....MKYVFI EKHQAEFSIK AMCRVLRVAR SGWYTWCQR. RTRISTRQQF
IS911     VRLPEQFSII GKLRAHYPVV TLCHVFGVHR SSYRYWKNR. PEKPDGRRAV
                    :     .   .    *  ..::  .:::    :  . :   * .

          51                                                 100
IS1141    TPTGCSRVHC PAAERAAILN ELHSERFIDT SPTEVWATLL DEGRYLGSIS
IS986     LKEHISRVH. .......... AA...NYGVY GARKVWLTLN REG.IEVARC
IS3       RQHCDSVVL. .......... AAFTRSKQRY GAPRLTDELR AQG.YPFNVK
IS911     LR...SQVL. .......... ELHGISHGSA GARSIATMAT RRG.YQMGRW
               * *                          ::  .   :   .* :

          101                                                150
IS1141    TFYRLLRQAG .....ESRER RRQATHPATV KP........ ELVAFEPNQV
IS986     TVERLMTKLG LSGTTRGKAR RTTIADPATA RP..ADLVQR RFGPPAPNRL
IS3       TVAASLRRQG LRA..KASRK FSPVSYRAHG LPVSENLLEQ DFYASGPNQK
IS911     LAGRLMKELG LVSCQQPTHR YKRGGHEHVA IP...NYLER QFAVTEPNQV
          :  ::..  *          :        :    *                **:.

          151                                                200
IS1141    WSWDITKLRG PAKWSWYYLY VILDIFSRYV VGWMVASRES AALA....EV
IS986     WVADLTYVST WA..GFAYVA FVTDAYARRI LGWRVASTMA TSMVLDAIEQ
IS3       WAGDITYLRT DE..GWLYLA VVIDLWSRAV IGWSMSPRMT AQLACDALQM
IS911     WCGDVTYIWT GK..RWAYLA VVLDLFARKP VGWAMSFSPD SRLTMKALEM
          *  *.* . .     .: *:  :..*...* . .** ...  . . :     :

          201                                                250
IS1141    LIRQTCAKQD IGRDRLTIHA DRGSSMTSKP VAFLLADLGV TQSHSRPHVS
IS986     AIWTRQQEGV LDLKDVIHHT DRGSQYTSIR FSERLAEAGI QPSVGAVGSS
IS3       ALWRRKRPR. ....NVIVHT DRGGQYCSAD YQAQLKRHNL RGSMSAKGCC
IS911     AWETRGKPG. ....GVMFHS DQGSHYTSRQ FRQLLWRYQI RQSMSRRGNC
           .              .  *. *:*:  :*       *    .   * :    .

          251                                                300
IS1141    DDNPFSEAQF KTLKYRPDFP D.RFDSIEAA RRHCQIFFGW YNDEHRHTGL
IS986     YDNALAETIN GLYKTELIKP GKPWRSIEDV ELATARWVD. WFNHRRLYQY
IS3       YDNACVESFF HSLKVECI.H GEHFISREIM RATVFNYIEC DYNRWRRHSW
IS911     WDNSPMERFF RSLKNEWI.P VVGYVSFSEA AHAITDYIVG YYSALRPHEY
           **.  *. : ..:*     :     . * :          .    . .  *

          301                                                350
IS1141    GLHVPADVHY GTAAIIRDKR AGVLDAAYAA HPERFVQKPP EPPKLPSGSW
IS986     CGDVPPVELE AAYYAQRQRP AAG*...... .......... ..........
IS3       CGGLSPEQFE NKNLA*.... .......... .......... ..........
IS911     NGGLPPNESE NRYWKKL*.. .......... .......... ..........
             .:.
```

FIG. 3.10. Alignment of the potential translation product of IS*1141* ORFb and the transposases of IS*986*, IS*3*, and IS*911*. Symbols show identity for IS*1141* with the other elements: (*), identity across all amino acid sequences; (:), two of three sequences are identical to IS*1141*; (.) at least two of three amino acid sequences share conservative substitution.

repeats (Kunze *et al.*, 1991), and IS1081 from *M. bovis* which has a IS*900*-like target site and 15 bp inverted repeats. The DNA sequences of the three cloned copies of the junction of IS*1141* with the surrounding DNA and a proposed target site of integration are listed in Fig. 3.7. Inspection of the DNA sequence surrounding the element suggested the possibility of 4 or 5 bp direct repeats (Fig. 3.7, bold italics) flanking the element. Nucleotides underlined in Fig. 3.7 show the target site similarity between IS*1141* and IS*900*. Unlike IS*1141*, IS*900* and IS*901* do not generate short direct repeats of the integration site, but because of the similarity of their terminal sequences and their target sequence, these elements do create a new target site as a result of insertion (Kunze *et al.*, 1991).

RFLP patterns of O and T variants. Since the opaque and transparent parental strains had different restriction fragment length polymorphism (RFLP) patterns, RFLP patterns of 16 Va14 colonial variants were determined by hybridization of *Sal*I digested genomic DNA with the *Apa*I-*Sma*I 1.5 kb IS*1141* probe (Fig. 3.11). The *Sal*I restriction fragments that originated from the plasmids were identified by including strain LV1 and variant LV26 total plasmid DNA samples on the Southern blot. pVT203 had a 4.4 kb band, pVT206 had a 2.6 kb band and pVT205 had 3.0 kb and 1.6 kb bands which hybridized to the IS*1141* probe. The O variants had 11 kb, 5.2 kb and 2.2 kb hybridizing bands from the chromosome and the T variants had 3.7 kb and 2.2 kb hybridizing bands in the chromosome. All O variants had a distinct chromosomal RFLP pattern compared to the T variants. The T variants all shared the same sized chromosomal bands and shared the 2.2 kb band with the O variants.

IS*1141* copy number. Since the opaque strain LV1 appeared to have either 4 or 5 copies of IS*1141* depending on the restriction endonuclease used (Fig. 3.5), the genomic DNAs

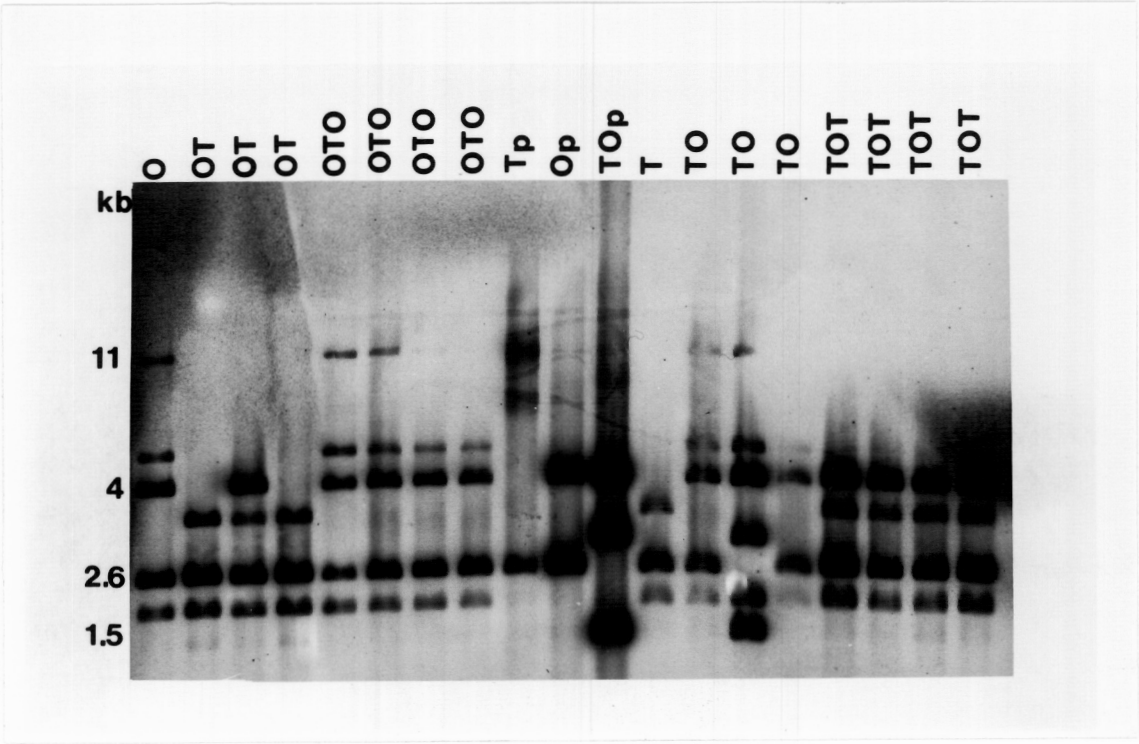

FIG 3.11. Hybridization of IS*1141* with total DNA from *M. intracellulare* strain Va14 colonial variants. The ^{35}S-labeled 1.5 kb *Apa*I-*Sma*I fragment of IS*1141* DNA was hybridized with *Sal*I-digested DNA from colonial variants of *M. intracellulare* strain Va14. Lanes are marked with the simplified strain designations listed in the nomenclature: O, opaque; T, transparent; OT, transparent revertant; TO, opaque revertant; TOT and OTO, are other combinations of reversions. The lanes are arranged so that the parental strain (O) or (T) is followed by the variants of that strain. The lanes containing *Sal*I digested plasmid DNA of LV2 (Tp), LV1 (Op), and LV26 (TOp) are indicated. The 1.5 kb *Sma*I-*Apa*I fragment of pVT328 served as the probe for hybridization. The apparent molecular masses (kb) of the DNA bands hybridizing to the probe are indicated at the left.

were digested with *Eco*RV which has a single central site in IS*1141* (Fig. 3.6) and probes to detect either the left [primers RI(+) and 1261(-)] or right end [primers LI(-) and 660(+)] of the IS element were prepared by PCR (see Fig. 3.6). When restriction endonucleases that did not cut IS*1141* were used (Fig. 3.5), there was a possibility that two copies of the element could be located on one restriction fragment or that two restriction fragments of the same size hybridized with the IS*1141* probe (Fig. 3.5). By cleaving the IS element into two fragments with *Eco*RV, each copy of IS*1141* was expected to be located on two different restriction fragments. So if Va14(O) contained five copies of IS*1141*, five bands would hybridize with the IS*1141* PCR probes. Had two copies of IS*1141* been present on one fragment in the head to tail orientation, the use of the right and left IS*1141* PCR probes on separate Southern blots, would still detect one band for each copy of the element present. This arrangement was predicted to detect if any IS*1141* copies were oriented such that two right ends or two left ends of the element were located on the same *Eco*RV fragment as well. If two right ends were present on the same fragment in the Va14(O) strain, the Southern blot probed with the right end probe would have four hybridizing bands and the Southern blot probed with the left end probe would have had five hybridizing bands. If two left ends of IS*1141* were present on one fragment, the opposite hybridization pattern would have been seen.

The 600 bp left-end probe hybridized to five bands in the opaque LV1 and three in the transparent LV2 (Fig. 3.12A). The O variants had a 1.2 kb hybridizing chromosomal band not found in the T variants. The 380 bp right-end probe hybridized with five bands in the opaque strain LV1 and three bands in the transparent strain LV2 (Fig. 3.12B). These results indicate that two copies of the right end or of two the left end of IS*1141*, were not present on the same

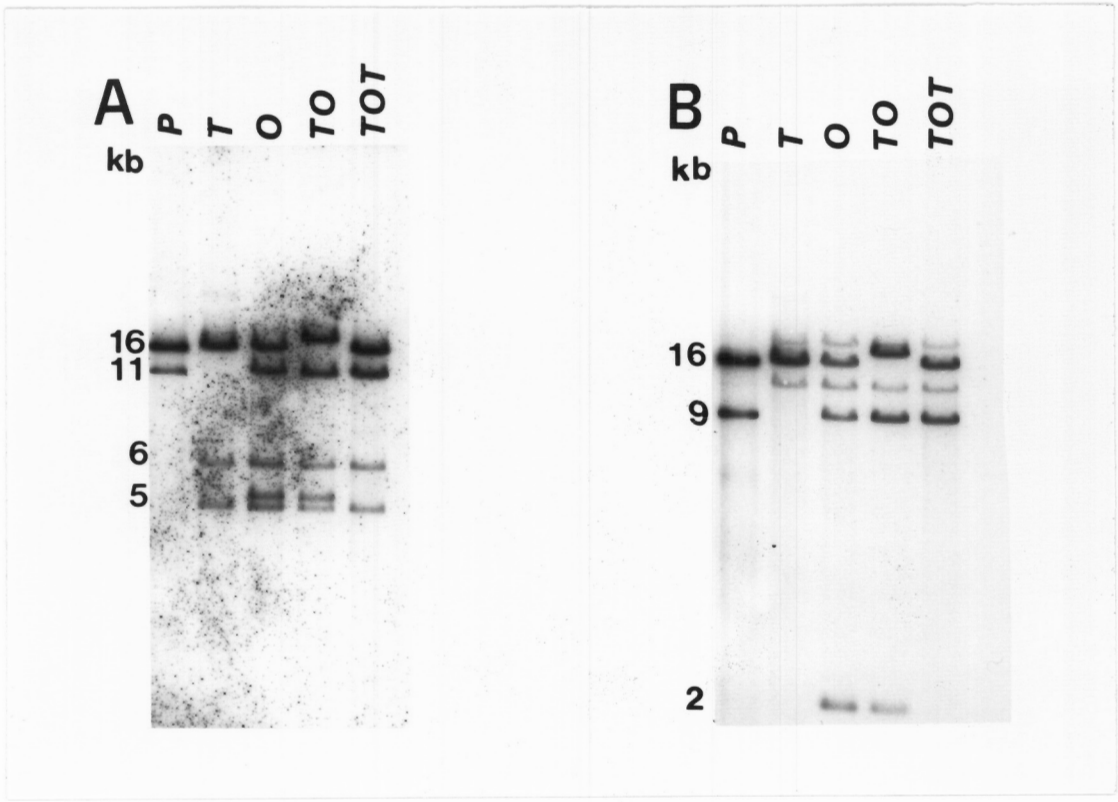

FIG 3.12. Copy number of IS*1141* in *M. intracellulare* strain Va14 colonial variants. Plasmid DNA of LV1 (P) and genomic DNAs of LV2 (T), LV1 (O), LV26 (TO) and LV29 (TOT) were digested with *Eco*RV which cleaves IS*1141* at nt 950, separated by electrophoresis, and transferred by Southern blotting. The Southern blot in panel A was probed with the PCR product of primers RI(+) and 1261(-) from the left side of IS*1141* (Fig. 3.6). The Southern blot in panel B was probed with the PCR product of primers LI(-) and 660(+) from the right side of IS*1141* (Fig. 3.6). The apparent molecular mass (kb) of the DNA bands hybridizing to the probes are indicated.

fragment. The left end of the IS*1141* copy from the 68 kb plasmid was located on a 11 kb fragment and the right end of that element copy was located on a 9 kb fragment. The 16 kb plasmid apparently has only one *Eco*RV site since both the left and right ends of that copy are located on a 16 kb fragment. None of the chromosome copies of IS1141 appear to be present on the same *Eco*RV fragment since the hybridizing fragments are all of different sizes. Transparent variants that contained the 68 kb plasmid had a fourth hybridizing band also found in O variants (Fig. 3.12B). The opaque variants had one 5 kb chromosomal band that hybridized with the right-end probe of IS*1141* not found in the T variants. The hybridization pattern suggests that the O and T variants may share two common chromosomal copies of the IS*1141* element rather than one as suggested by the Southern blot prepared with *Sal*I (Fig. 3.11).

Growth profiles of variants. The opaque strain LV1 grew faster than its T derivative LV11 in M7H9 without Tween 80 (Fig. 3.13). When Tween 80 was added to 0.5%, the growth rates of variants were similar. Little measurable growth of variant LV11 was observed in other mycobacterial media without adding fatty acids (data not shown). Only in the 0.5% Tween 80-containing M7H9-based medium above did variant LV11 approach the growth rate and turbidity of variant LV1. The growth response of variant LV11 and the parental transparent variant LV2 were identical as were the responses of strain LV1 and O variant LV21 (data not shown).

Antibiotic susceptibilities. The transparent variants, LV2 and LV11, were more resistant to imipenem, some of the tested β-lactam antibiotics and the cephlosporins than the opaque strain LV1 and variant LV21 (Table 3.2). Of the 19 antibiotics tested, the T variants

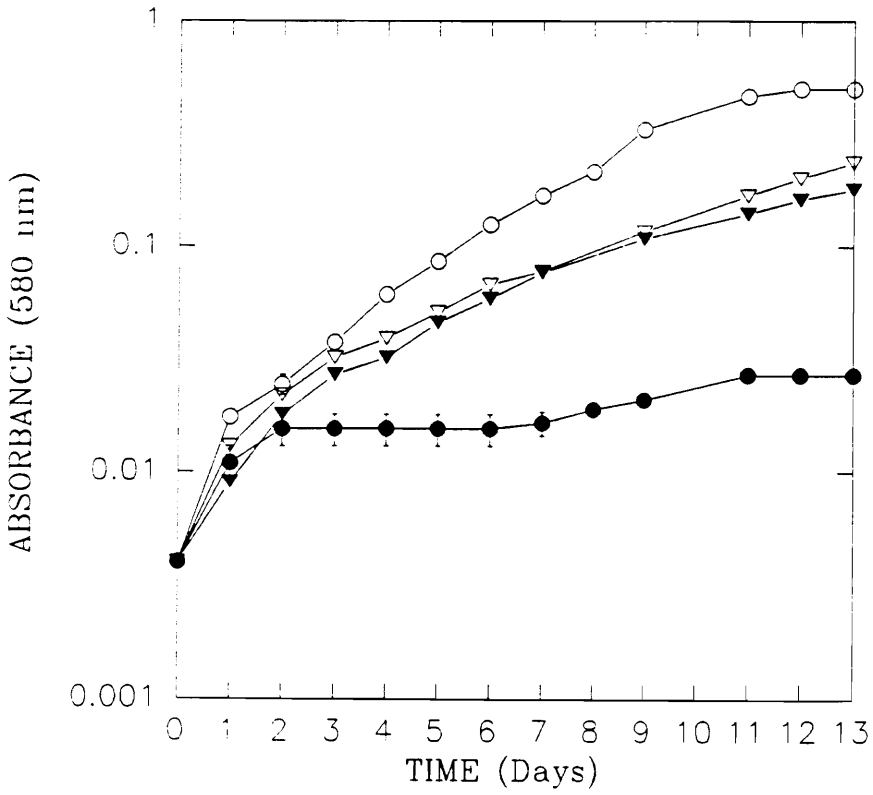

FIG 3.13. Growth response of *M. intracellulare* Va14 variants to Tween 80. The growth of *M. intracellulare* opaque strain LV1 (▼) and transparent variant LV11 (●) in Middlebrook 7H9 medium containing 10% ADC and LV1 (▽) and LV11 (○) in M7H9 medium containing 10% ADC and 0.5% Tween 80 at 37° C. Growth was measured by the increase in absorbance at 580nm.

Table 3.2. Antibiotic susceptibility (as MIC) of *M. intracellulare* strain Va14 opaque and transparent colonial variants using Sceptor System microdilution panels.

Antibiotic	MIC (μg/ml)			
	LV1(O)	LV11(OT)	LV2(T)	LV21(TO)
Aminoglycosides				
Amikacin	8	8	8	8
Gentamicin	4	4	8	4
Tobramycin	8	8	8	8
Vancomycin	> 32	> 32	> 32	16
β-Lactams				
Penicillin	> 16	> 16	> 16	16
Ampicillin	> 16	> 16	> 16	8
Carbenicillin	8	>128	>128	4
Piperacillin	< 4	>256	>256	4
β-Lactams + β-Lactamase Inhibitor				
Augmentin	> 8/4	> 8/4	> 8/4	8/4
Unasyn	16/8	>32/16	>32/16	16/8
Cephalosporins				
Ceftizoxime	< 4	>256	>256	16
Ceftriaxone	4	32	128	16
Cefoperazone	32	64	64	32
Ceftazidime	32	> 64	> 64	32

Table 3.2. continued

Antibiotic	MIC (μg/ml)			
	LV1(O)	LV11(OT)	LV2(T)	LV21(TO)
Quinolones				
Ciprofloxacin	4	4	4	4
Norfloxacin	> 16	16	16	8
Imipenem	< 8	> 64	> 64	16
Aztreonam	32	> 32	> 32	32
Rifampin	4	2	2	4

were less susceptible to eight of the drugs using the Sceptor system. Both O and T variants were resistant to the highest drug concentration of oxacillin (8 μg/ml), clindamycin (8 μg/ml), and erythromycin (8 μg/ml) present on the Sceptor panel.

Rate of Variation. The appearance of transparent variants in an opaque culture was measured as between 10^{-6} and 10^{-8} per cell per generation in early log phase cultures. The appearance of O variants was approximately 10^{-4} per cell per generation. The wide ranges given are a function of the difficulty in measuring rates of variation of two colonial types with differing growth rates in MGE broth culture. Tween 80-containing media enhance the growth rate of T variants making the generation time of both variants more equal, but Tween 80 makes T variants susceptible to D-cycloserine used to inhibit the growth of O variants and to detect T variants.

DISCUSSION

During an investigation of the mechanism of colonial variation in *M. avium* complex, an insertion sequence, IS*1141,* was discovered in a 68 kb plasmid of the non-AIDS clinical *M. intracellulare* strain Va14. The investigation determined that O and T variants of strain Va14 matched the classic definition of interconvertable O and T colonial variants established for the *M. avium* complex (Scheafer *et al.*, 1970; Woodley and David, 1976; Kajioka and Hui, 1978; Kuze and Uchihira, 1984). The estimate of rates of variation for the two interconvertable variants of Va14(O) and Va14(T) agree with those calculated by McCarthy (1970) and Woodley and David (1976). Strain Va14 transparent and opaque variants had growth patterns consistent (Fig. 3.13) with those observed by Kajioka and Hui (1978). In addition, the T variants of Va14 were observed to be generally less susceptible to antibiotic treatment than the Va14 opaque variants (Table 3.2) similar to the strains investigated by Kuze and Uchihira (1984). Some Va14 transparent variants had a different plasmid profile (240, 62, 25, and 16 kb) than the O variants of the strain (240, 68, 25, and 16; Fig. 3.1).

The change in plasmid profile of the strain Va14 variants initially appeared associated with a concurrent change in the colony morphology, particularly since no change in plasmid profile was detected in the sibling O or T variants inspected. Subsequent inspection of the plasmid profiles of many independent O and T variants identified the presence of the 68 kb plasmid in OT, TO, OTO, and TOT variants (Table 3.1). The rarer 62 kb plasmid was only seen in the T parent strain LV2 and OT variants LV11 and LV115.

In an effort to isolate the putative element, DNA fragments from pVT203 (68 kb) and

pVT204 (62 kb) were cloned and restrictions maps were developed (Fig. 3.2 and 3.3). A 3.3 kb region of DNA present in pVT203 but not in pVT204 was found to hybridize with the 68, 18, and 16 kb plasmids. An IS element, designated IS*1141*, was found in this DNA fragment of the 68 kb plasmid of strain Va14(O) that was absent in the 62 kb plasmids (Fig. 3.4) of transparent variants LV2, LV11 and LV115. The 16 kb plasmid with one copy of IS*1141* was found in both O and T variants (Fig. 3.4). The 18 kb plasmid containing two copies of IS*1141* has been observed in one O variant (Fig. 3.4 and 3.11) and one rough variant not discussed in this study. Because the 68 kb plasmid was observed in both T and O variants (Table 3.1) and since IS*1141* was present in the 16 kb plasmid in all variants, the presence of IS*1141* in the plasmids appears to be unrelated to colonial variation of strain Va14.

Flanking the unique region containing IS*1141* in pVT203, a 1.8 kb region of DNA appeared to be repeated. Restriction digests of total plasmid DNA from the 68 kb plasmid-containing variants indicated that this duplication had been generated in each of the seven independent variants (Table 3.1) showing a 62 to 68 kb plasmid change. Restriction analysis and hybridization studies have shown that only one copy of this 1.8 kb region was present in pVT204 (62 kb) and the region was not in the 16 or 18 kb plasmids (data not shown). If this duplication occurred with the transposition of IS*1141*, and large duplications surrounding the IS*1141* element insertion site occur with transposition, the element will prove to be quite unusual for a prokaryotic mobile element. No investigation of the flanking regions of IS*1141* in the 16 or 18 kb plasmids has been undertaken.

In addition to the 68 kb and 16 kb plasmid copies of IS*1141*, the chromosome of Va14 transparent variants contains two copies and the chromosome of O variants contains three

copies of IS*1141* (Fig. 3.5). In the *Sal*I restriction digest, O variants each share a pair of hybridizing bands that are not present in the T variants of strain Val4 (Fig. 3.11). Each of seven O variants had the same chromosomal IS*1141* RFLP pattern as the parental opaque strain LV1 and of seven T variants each showed the same chromosomal IS*1141* RFLP pattern as the parental T but in each set various plasmid patterns were observed. The position of IS*1141*'s in the chromosome of the Val4 variants is significant because the RFLP pattern of IS*1141* correlates with colony morphology (Fig. 3.5, 3.11 and 3.12).

Sequence comparison of the 16 kb plasmid and chromosome fragments containing IS*1141* was initiated because the 0.5 kb *Pvu*II-*Apa*I and 1.5 kb *Apa*I-*Sma*I subclones sequenced from the 68 kb plasmid did not have obvious inverted and direct repeats. The IS element identified (Fig. 3.7 and 3.8) was determined to have imperfect inverted repeats and appeared to generate direct repeats upon transposition (Fig. 3.7). Two IS*900* copies from the one *M. paratuberculosis* strain (Green *et al.*, 1989) had identical sequences, but IS*986* and IS*6110,* which are the same element from different *M. tuberculosis* strains, contained 13 nt changes over the 1358 bp sequence. Comparison of the sequences of the three IS*1141* copies cloned from strain LV1 indicated that the copies were identical in the regions sequenced.

IS*1141* was 1596 bp in length (Fig. 3.8) with 7 ORF's, (Fig. 3.9). Data base searches and a multiple sequence alignment (Fig. 3.10) indicate that IS*1141* ORFb has significant similarity to the IS*3* family transposases (Prere *et al.*, 1990) and IS*986*, an IS*3*-like element (McAdam *et al.*, 1990), from *M. tuberculosis*. Nucleotide and putative amino-acid sequence similarities between the other *M. avium* complex (IS*900,* IS*901,* and IS*902*) IS elements are insignificant, but IS*1141* does have a similar target insertion sequence (Fig. 3.7) to IS*900*

(Green *et al.*, 1989) and IS*901* (Kunze *et al.*, 1991) as well as IS116 from *Streptomyces clavigerus* (Leskiw *et al.*, 1990). Although the ends of IS*1141* somewhat resemble the ends of these atypical elements, it is not clear whether the IS*900*-like ends, the IS*3*-like inverted repeats of IS*1141*, or both are involved in transposition.

In reviewing the structure of IS*3*, IS*911*, and IS*986*, each has a poor Shine-Dalgarno sequence preceding ORFb (Prere, *et al.*, 1990; McAdam *et al.*, 1990) similar to IS*1141*. In IS*3* and IS*3411*, the translational stop signal of ORFa overlaps the first likely start codon of ORFb in the sequence ATGA. In IS*1141*, ORFa terminates with TGA (position 534) and ORFb begins with GTG at position 536 (**TGA**AGTG; Fig. 3.8). IS*1141* ORFc overlaps ORFb in a -1 orientation in the sequence GTGA like the overlap in IS*3* (Fig. 3.8) This arrangement suggests ribosomal read-through or re-initiation as a possible mechanism for translation of ORFb (Prere *et al.*, 1990; McAdam *et al.*, 1990). The structure of IS*986* is similar in that two small ORFs proceed ORFb but the termination of ORFa2 and the beginning of ORFb overlap by approximately 100 bp. McAdam *et al.*, (1990) have suggested translational frameshifting as the mechanism of translation of IS*986* ORFb. Recently Polard *et al.* (1992) have demonstrated that translational frameshifting occurs in IS*911* producing a fused ORFa-ORFb protein capable of mediating transposition. IS*1141* lacks the adenine and uracil rich region upstream from ORFb thought to be involved in translational frameshifting (Polard *et al.*, 1992), so if IS*1141* produces a fused ORFc-ORFb protein product, it is likely to occur by ribosome read-through of the termination codon of the mRNA.

Most of the IS*3* family elements have an ORF on the reverse strand in the transposase coding region (Galas and Chandler, 1989; Prere *et al.*, 1990). IS*1141* has two small ORFs in

that region which are likely to be transcribed (Fig. 3.9). Several IS elements, including IS*3*, have been observed to activate transcription of genes downstream from their insertion site due to outward orientated promoters or the formation of new promoters using an outward orientated (-)35 promoter region (Galas and Chandler, 1989). IS*1141* has sequences similar to outward oriented promoters (Galas and Chandler, 1989; Lewin, 1990) located at both ends of the element (Fig. 3.8) as well as outward oriented (-)35 promoter regions. Due to the similarities between IS*1141* and the IS*3*-like elements IS*911* and IS*986*, we propose IS*1141* to be a member of the IS*3* family.

Upon integration in a gene, IS elements often cause changes in gene expression (Galas and Chandler, 1989). In general, because of the pattern of loss of pathogenicity, antibiotic resistance, and T-specific antigens (Thorel and David, 1984) it has been assumed that the T phenotype requires transcription of gene(s) not active in O variants and that O variants are not necessarily transcribing genes not active in the T variants, but there is little evidence on which to base this assumption. Since the Va14 opaque variants have an additional chromosomal copy of IS*1141* absent in the T variants (Fig. 3.5, 3.11, and 3.12), the mechanism of colonial variation in this strain could be as simple as IS*1141*-generated insertional activation (Fig. 3.14, model 1) that turns off expression of the gene or genes necessary for the T phenotype. Transparent variants have various resistance and pathogenicity characteristics which could be caused by additional amounts or types of surface glycolipids not present in O variants (David *et al,* 1987). Yet, if that simple mechanism were the explanation of colonial variation in all strains, IS*1141* or another transposable element would be required for interconversion. The rate of perfect excision of transposable elements from the chromosome is low,

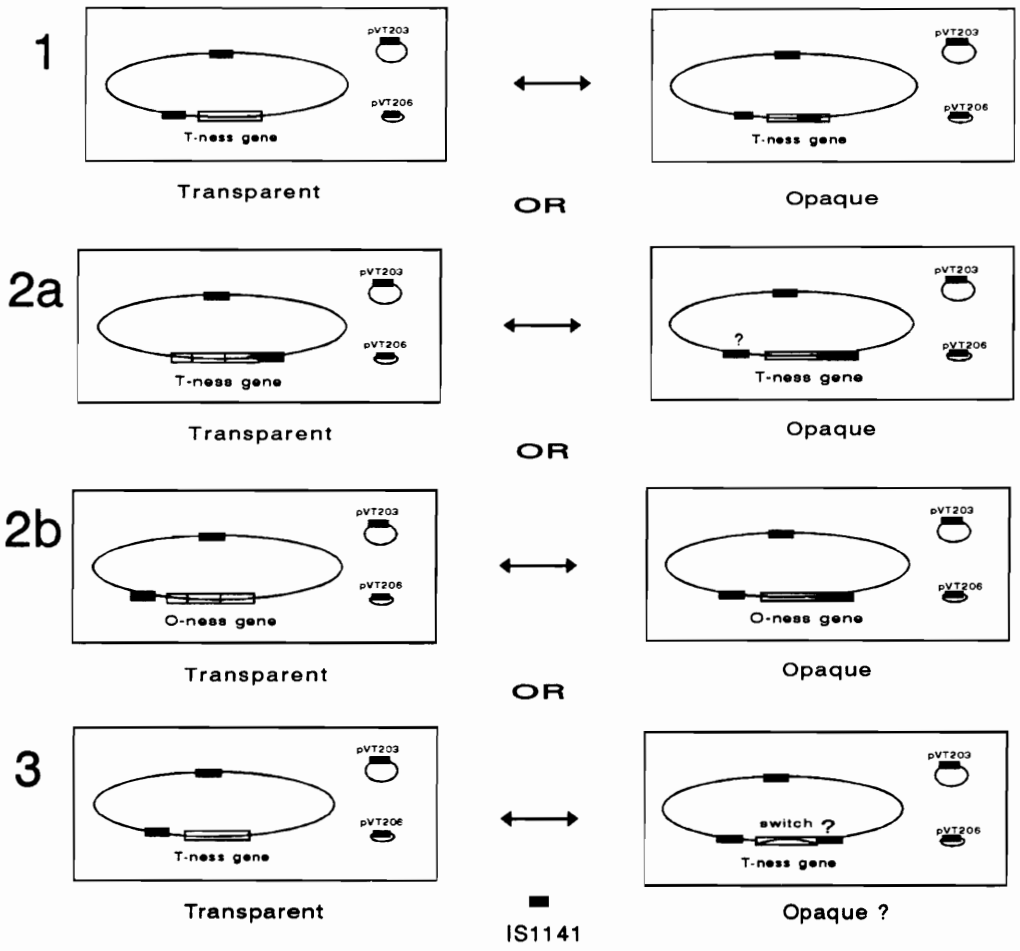

Fig. 3.14. Models for the mechanism of colonial variation in *M. intracellulare* strain Va14. Each model is depicted as two cells, one from a transparent (T) colony and one from an opaque (O) colony, each containing a chromosome (large oval), two plasmids (small ovals labeled pVT203 and pVT206), a DNA region responsible for the T or O phenotype (open rectangle on the chromosome, labeled T-ness or O-ness gene), and copies of IS*1141* (small solid rectangles). Model 1 shows IS*1141*-mediated insertional inactivation, Model 2a and 2b show IS*1141* providing a promoter for the region responsible for colony phenotype, and model 3 shows a molecular switch in the T-ness region with IS*1141* located close by, such that the alternation of the switch would change the size of restriction fragments containing IS*1141*.

10^{-6} to 10^{-9}/cell/generation depending on the element (Ehrlich, 1989) suggesting the rate of reversion to a T phenotype would be low, yet similar to the reversion rate we estimated for strain Va14. But if IS*1141* creates large 1 to 2 kb duplications in the target DNA during integration, the expected rate of reversion to the T phenotype would be much higher than we have observed, since rates for homologous recombination are on the order of 10^{-4} /cell/generation (Galas and Chandler, 1989).

If IS*1141* is not disrupting transcription of the gene(s) responsible for the T phenotype, then it is possible that IS*1141* is providing a promoter for genes coding for the T phenotype (Fig. 3.14, model 2a) or the possibly undetected gene(s) necessary to produce the O phenotype (Fig. 3.14, model 2b). The RFLP patterns generated with *Eco*RV (Fig. 3.12) do not support the IS*1141* promoter T phenotype model (Fig. 3.12) because the RFLP patterns suggest the T variants have IS*1141* in the same DNA fragments as O variants and that O variants have an additional copy of IS*1141*. If the IS is providing a promoter for genes for the T phenotype, then O variants without the third chromosomal copy of IS*1141* should exist. On the other hand, the RFLP pattern data can support the model of IS*1141* providing a promoter for O phenotype genes (Figs. 3.5, 3.12, 3.14 model 2b).

A third possibility (Fig. 3.14, model 3) is that IS*1141* has one or two target insertion sites in or near the DNA region responsible for colonial variation. When this region undergoes some type of rearrangement, (*e.g.*, inversion or duplicative transposition), the bacterium forms the alternate colony type and one or two of the IS*1141*-containing DNA fragments is changed or duplicated, resulting in the changed RFLP pattern observed (Fig. 3.11 and 3.12). Since DNA that hybridizes with IS*1141* has been observed in other *M.*

intracellulare strains, but not in all MAC strains showing colonial variation (Chapter 4), mechanisms which do not require insertion sequences need to be considered.

The data available are most completely explained by the IS1141-generated polarity in gene(s) expressing the T phenotype. Investigation of the DNA flanking the IS*1141* copies should identify a gene or operon being interrupted by IS*1141* or possibly a gene or genes being transcribed from one of IS*1141*'s outward oriented promoters. If neither of these are found, a search for DNA flanking the insertion sites which when used as a probe, also gives different RFLP patterns between T and O variants should be initiated. In addition, because IS*1141* transposition can be detected and measured, a study of the mechanism of transposition in mycobacteria can be initiated.

CHAPTER IV

Distribution of IS*1141* in clinical and environmental isolates of the *Mycobacterium avium* complex

ABSTRACT

Insertion sequence IS*1141* was discovered in the 68 kilobase plasmid of the clinical non-AIDS *Mycobacterium intracellulare* strain Va14. The IS element is 1596 base pairs in length with 23 bp imperfect terminal inverted repeats. A survey of 32 Virginia clinical, non-AIDS *Mycobacterium avium* complex isolates identified two of 19 *M. intracellulare* isolates, Va2 and Va10, which also contained IS*1141*. *M. intracellulare* isolates Va2, Va10, and Va14 were recovered from patients who were from localities near the James River, Virginia. A survey of 31 environmental *M. avium* complex isolates from James River aerosols or water identified one of four *M. intracellulare* isolates as containing IS*1141*. IS*1141* has not been detected in any clinical or environmental *M. avium, Mycobacterium scrofulaceum,* or *Mycobacterium* species X isolates to date. IS*1141* appears to be limited to the *M. intracellulare* species, but it is present in only 17% of the *M. intracellulare* isolates surveyed. The IS*1141* restriction fragment length polymorphism pattern of each isolate was unique.

INTRODUCTION

Mycobacterium avium has been the predominant *M. avium* complex (MAC) species isolated from human immunodeficiency virus (HIV)-infected patients (Wolinsky, 1992). *Mycobacterium intracellulare* infection of HIV-infected patients was thought to be rare (Yakrus and Good, 1990), but Cregan *et al.* (1992) recently reported 10 *M. intracellulare* isolates among 93 consecutive MAC isolates from HIV-seropositive patients. Representatives of MAC are responsible for bacteremia in approximately 25% of acquired immunodeficiency syndrome (AIDS) patients (Blaser and Cohen, 1986; Horsburgh *et al.*, 1991) and increasing rates of pulmonary disease in non-AIDS patients (Iseman, 1989). Because of the widespread occurrence of MAC bacteria in natural waters (Falkinham *et al.*, 1980; Kirschner *et al.*, 1992), municipal water supply systems (DuMoulin and Stottmeier, 1986), hospital hot water supply systems (DuMoulin *et al.*, 1988), soils (Brooks *et al.*, 1984) and aerosols (Wendt *et al.*, 1980) and lack of evidence of person-to-person transmission of *M. avium* (Wolinsky, 1992), the source of MAC inoculum for humans is thought to be the environment. Identification of the source of MAC inoculum requires a typing system for MAC species which has sufficient discriminatory power to distinguish between individual isolates and demonstrate the identity of environmental and clinical isolates.

In *Mycobacterium tuberculosis,* multiple copies of the insertion sequence IS*6110* are found (Thierry *et al.*, 1990a). Because the position of IS*6110* insertions vary from isolate to isolate (Hermans *et al.*, 1990), restriction fragment length polymorphism (RFLP) patterns of IS*6110* can serve as fingerprints for individual *M. tuberculosis* isolates (Cave *et al.*, 1991;

Mazurek *et al.*, 1993). In *Mycobacterium bovis*, another member of the *M. tuberculosis* complex, fewer copies of IS*6110* are present and only a few RFLP patterns are found among *M. bovis* isolates (van Soolingen *et al.*, 1991; Fomukong *et al.*, 1992). The presence of IS*6110* was found to be diagnostic for *M. tuberculosis, M. bovis, Mycobacterium africanum,* and *Mycobacterium microti* (Thierry *et al.*, 1990b), but the element only appears to be useful in differentiating among isolates in *M. tuberculosis.*

To date, no insertion sequence detected in *M. avium* complex pathogens is present in all members of the complex. Although the insertion sequence IS*900* (Green *et al.*, 1989) has been found in every isolate of *Mycobacterium paratuberculosis* (Vary *et al.*, 1990), only a limited number of RFLP patterns have been observed (McFadden *et al.*, 1987) and thus IS*900* is diagnostic for the species but not useful for typing. The *M. avium* insertion sequence IS*901* (Kunze *et al.*, 1991) is found in *M. avium* isolates from animals and has been used to divide the *M. avium* species into subgroups (Kunze *et al.*, 1992). If these subgroups are artificial or not remains to be seen. RFLP analysis using IS*901* as a probe showed that *M. avium* isolates from all of 81 animals had IS*901* while only, four of 44 non-AIDS patients and none of eight environmental samples contained the element (Kunze *et al.*, 1992; J.J. MaFadden, personal communication). Although IS*901* may be useful in identifying *M. avium* subtypes, it also does not have the power to discriminate among isolates. IS*900* and IS*901* (and its close relative IS902; Moss *et al.*, 1992) have not been found in *M. intracellulare.* Because IS*1141* was the first insertion sequence reported in *M. intracellulare*, we sought to determine if IS*1141* could be useful as a diagnostic tool and if individual MAC isolates have unique IS*1141* RFLP patterns.

In order to evaluate the usefulness of IS*1141* as an RFLP marker, two *M. avium* complex strain collections were surveyed for the presence of IS*1141*. One collection consisted of pulmonary isolates from non-AIDS patients residing in the James River basin of Virginia. The second collection consisted of James River water and aerosol isolates collected along the river from Richmond to Hampton Roads (Falkinham *et al.*, 1980; Wendt *et al.*, 1980; Fry *et al.*, 1986; Falkinham *et al.*, 1990). The isolates were previously identified as MAC bacteria, but the methods used at the time could not distinguish among MAC members. In this study, the SNAP System (Syngene, Inc., San Diego, CA) was used to identify isolates in the collections to species. Hybridization studies were used to identify isolates which contained IS*1141* and three other recently used MAC epidemiologic markers, IS*901* (Kunze *et al.*, 1991), plasmid pLR7 (Crawford and Bates, 1986) and plasmid pVT2 (Jucker and Falkinham, 1990).

The results suggest that, like IS*901*, IS*1141* is present in only some *M. avium* complex isolates. IS*1141* has been detected only in *M. intracellulare* isolates and may be limited to that species. It was present in 15% of clinical and 25% of environmental *M. intracellulare* isolates in the plasmid and chromosome replicons.

METHODS AND MATERIALS

Mycobacterial strains. Thirty-three *M. avium* complex pulmonary isolates (Table 4.1) from Virginia non-AIDS patients who lived in the James River basin were obtained as primary isolates from Dr. Nancy Warren, Consolidated Laboratories, Richmond VA. Thirty-one environmental MAC isolates (Table 4.2) were recovered from water (Fry *et al.*, 1986; Falkinham *et al.*, 1990), and aerosols (Wendt *et al.*, 1980) collected in the James River basin as described. *M. intracellulare* strain pairs [Va2-11 and Va2-12; Va10-33 and Va10-63; Va14(O) and Va14(OT)] with differing plasmid profiles (Erardi *et al.*, 1985; Via and Falkinham, 1991a) were examined for the presence of IS1141, as were the type strains of *M. avium* (TMC724^T), *M. intracellulare* (TMC1406^T), and *Mycobacterium scrofulaceum* (TMC1323^T).

Sources of epidemiologic markers. The sources of IS*1141*, IS*901*, pVT2, and pLR7 are listed in Table 4.3. *M. intracellulare* strain Va14(O), carrying pVT203, was the source of IS*1141* (Fig. 4.1) which was cloned into pBluescriptSK (Stratagene, La Jolla, CA) yielding pVT365 (Fig. 4.1). IS*901* was cloned in plasmid pUS410 from *M. avium* strain FP8589 (Kunze *et al.*, 1991). pVT2 is a 12.9 kb plasmid from *M. intracellulare* strain MD1 cloned in two pieces (pVT100 and pVT101) into pUC19 and used to transform *Escherichia coli* strain JM109 (Jucker and Falkinham, 1990). The 15.3 kb plasmid pLR7 was cloned from *M. avium* LR113 into pJC20 (Crawford and Bates, 1986).

Isolation and radiolabeling of DNA probes. DNA fragments of IS*1141* (Fig. 4.1), IS*900*, pLR7, and pVT2 were released from recombinant plasmids (Table 4.3) by restriction

Table 4.1. *Mycobacterium avium* complex pulmonary isolates from Virginia non-AIDS patients.

Isolate	Location[a]	Species[b]	Hybridization profile				Other Plasmids[c]	Reference[d]
			IS*1141*	IS*901*	pLR7	pVT2		
Va1	Richmond	MA	-	-	-	-	No	A
Va2	Richmond	MI	+	-	-	+	Yes	A
Va3	Richmond	MA	-	-	+	+	Yes	A
Va4	Richmond	MA	-	-	-	-	No	A
Va5	Richmond	MI	-	-	-	+	Yes	A
Va6	Richmond	MI	-	-	-	-	Yes	A
Va7	Petersburg	MA	-	-	-	-	No	A
Va8	Petersburg	MA	-	-	-	-	No	A
Va9	Hampton	MI	-	-	-	-	Yes	A
Va10	Hampton	MI	+	-	-	+	Yes	A
Va11	Hampton	MX	-	-	-	-	No	A
Va12	Hampton	MA	-	-	+	-	Yes	A
Va13	Hampton	MA	-	-	-	-	No	A
Va14	Norfolk	MI	+	-	-	-	Yes	A
Va15	Norfolk	MI	-	-	-	-	Yes	A
Va16	Norfolk	MI	-	-	-	-	No	A
Va17	Portsmouth	MAC	-	-	-	-	No	A
Va18	Va. Beach	MAC	-	-	-	-	Yes	A
Va19	Colonial Hts.	MI	-	-	-	-	No	A

Table 4.1, continued.

Isolate	Location[a]	Species[b]	Hybridization profile				Other Plasmids[c]	Reference[d]
			IS*1141*	IS*901*	pLR7	pVT2		
Va20	Colonial Hts.	MI	-	-	-	-	No	A
Va21	Accoma	MAC	-	-	-	-	Yes	A
Va22	Hayes	MI	-	-	-	-	Yes	A
Va23	N. Warren	MA	-	-	+	+	Yes	B
Va24	N. Warren	MA	-	-	-	-	Yes	B
Va25	N. Warren	MI	-	-	-	-	Yes	B
Va26	N. Warren	MI	-	-	-	-	Yes	B
Va27	N. Warren	MI	-	-	-	-	Yes	B
Va28	N. Warren	MI	-	-	-	-	Yes	B
Va29	N. Warren	MI	-	-	-	-	Yes	B
Va30	N. Warren	MI	-	-	-	-	No	B
Va31	N. Warren	MI	-	-	-	-	Yes	B
Va32	N. Warren	MI	-	-	-	-	No	B
Va33	N. Warren	MI	-	-	-	-	Yes	B

[a] Location of patient's home or hospital, N. Warren listed if data unavailable. [b] MA, *M. avium*; MI, *M. intracellulare*; MX, *Mycobacterium* species "X" as defined by the SNAP System (Syngene, Inc., San Diego, CA); MAC, *M. avium* complex by cultural and biochemical characteristics, but SNAP DNA-probe negative. [c] Meissner and Falkinham, 1986. [d] Reference A, Fry *et al.*, 1986; reference B, Meissner and Falkinham, 1986.

Table 4.2. Environmental *M. avium* complex isolates from the James River basin in Virginia.

Isolate	Location	Sample[a]	Species[b]	Hybridization profile				Other Plasmids[c]	Reference[d]
				IS*1141*	IS*901*	pLR7	pVT2		
1S	Richmond	Aerosol (3)	MA	-	-	-	-	Yes	A
2S	Richmond	Aerosol (5)	MA	-	+	+	-	Yes	A
4S	Richmond	Aerosol (5)	MI	+	-	-	+	Yes	A
5S	Richmond	Aerosol (1)	MA	-	-	-	+	Yes	A
8S	Richmond	Aerosol (4)	MA	-	-	-	-	Yes	A
9S	Richmond	Aerosol (6)	MAC	-	-	-	-	No	A
10S	Richmond	Aerosol (4)	MA	-	-	-	-	Yes	A
11S	Richmond	Aerosol (2)	MA	-	-	+	-	Yes	A
12S	Richmond	Aerosol (3)	MA	-	-	+	-	Yes	A
13S	Richmond	Aerosol (3)	MA	-	-	-	-	No	A
14S	Richmond	Aerosol (3)	MX	-	-	-	-	No	A
JR074	Richmond	Aerosol (6)	MX	-	-	-	-	No	B
JR129	Richmond	Water	MA	-	-	-	-	No	B
JR223	Richmond	Water	MAC	-	-	-	-	No	B
JR326	Richmond	Water	MX	-	-	-	-	No	B
JR584	Richmond	Water	MI	-	-	-	-	No	B
JR686	Richmond	Water	MI	-	-	-	-	No	B
JR820	Richmond	Water	MAC	-	-	-	-	No	B
JR959	Richmond	Water	MI	-	-	-	-	No	B

Table 4.2. continued.

Isolate	Location	Sample[a]	Species[b]	Hybridization profile				Other Plasmids[c]	Reference[d]
				IS*1141*	IS*901*	pLR7	pVT2		
JR1088	Richmond	Water	MX	-	-	-	-	No	B
JR1360	Richmond	Aerosol (2)	MA	-	-	-	-	No	B
JR1361	Richmond	Aerosol (2)	MX	-	-	-	-	No	B
JR1385	Richmond	Aerosol (5)	MX	-	-	-	-	No	B
JR1388	Richmond	Aerosol (4)	MX	-	+	-	-	No	B
JR1394	Richmond	Water	MAC	-	-	-	-	No	B
JR1470	Richmond	Aerosol (2)	MAC	-	-	-	-	No	B
W33	Newport News	Water	MX	-	+	-	-	No	C
W266	Claremont	Water	MX	-	-	-	-	No	C
W277	Claremont	Water	MA	-	-	-	-	No	C
W280	Newport News	Water	MAC	-	-	-	-	No	C
W284	Claremont	Water	MA	-	-	-	-	No	C

[a] Anderson sampler stage (numbers 1 to 6) from which isolate was recovered (Anderson, 1958) is given for aerosol samples. [b] MA, *M. avium*; MI, *M. intracellulare*; MX, *Mycobacterium* species "X" as defined by the SNAP System (Syngene, Inc., San Diego, CA); MAC, *M. avium* complex by cultural and biochemical characteristics, but SNAP DNA-probe negative. [c] Meissner and Falkinham, 1986. [d] Reference A, Wendt *et al.*, 1980; reference B, Falkinham *et al.*, 1990; reference C, Fry *et al.*, 1986.

Table 4.3. Sources of IS elements and plasmids used as DNA hybridization probes in this study

Marker	Origin	Recombinant source	Probe fragments	Reference
IS*1141*	*M. intracellulare* Va14(O) plasmid pVT203	pVT365 in *E. coli* XL1	1.5 kb *Bst*EII-*Eco*NI	Via and Falkinham, in prep.
IS*901*	*M. avium* FP8589 goat isolate	pUS410 in *E. coli*	0.8 kb *Hin*cII	Kunze *et al.*, 1990
pLR7	*M. avium* LR113 pLR7	pJC20 in *E. coli* HB101	15.3 kb *Hin*dIII	Crawford and Bates, 1984
pVT2	*M. intracellulare* MD1 pVT2	pVT100 and pVT101 in *E. coli* JM109	4.2 kb *Eco*RI-*Hin*dIII and 8.7 kb *Eco*RI-*Hin*dIII	Jucker and Falkinham, 1990

endonuclease digestion and separated from the remaining DNA fragments by electrophoresis in SeaPlaque GTG agarose (FMC BioProducts, Rockland, ME). These restriction fragment bands and the IS*1141* 1550 bp PCR product (Fig. 4.1) were radiolabeled in-gel with α-^{35}S-dCTP (1,000 Ci/mmol) or [α^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Cambridge, MA) using instructions provided with the random-primed DNA labeling kit (Boehringer-Mannheim, Inc., Indianapolis, IN).

Growth of mycobacteria. Strains were maintained on Middlebrook (M) 7H10 agar (BBL Microbiology Systems, Cockeysville MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin (OA) enrichment (Pethel and Falkinham, 1989). Stock broth cultures of all strains inoculated from slants were incubated at 37° C in 2 ml M7H9 broth base (BBL Microbiology Systems, Cockeysville MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin (OA) enrichment (MGE broth) (Pethel and Falkinham, 1989) for 4 to 7 d. Two ml of those stock cultures were used to inoculate 10 ml of MGE broth and the cultures incubated at 37° C with rotation (10 RPM, TC-6 Rollordrum, New Brunswick Scientific, Edison, NJ) until they reached the turbidity of a 0.5 to 1 McFarland standard (7 to 35 d depending on the isolate). Growth of mycobacteria for plasmid or large scale genomic DNA isolation is described with the isolation method.

Dot blot hybridization optimization. In order to screen the clinical and environmental isolates for presence of IS*1141*, a method which would allow detection of one or two copies of IS*1141* per genome was required. *M. intracellulare* strains containing IS*1141* in both the plasmids and the chromosome [Va14(O) and Va14(T)] and only in the chromosome [Va10-63] were used in the dot blot optimization. Four methods for preparing

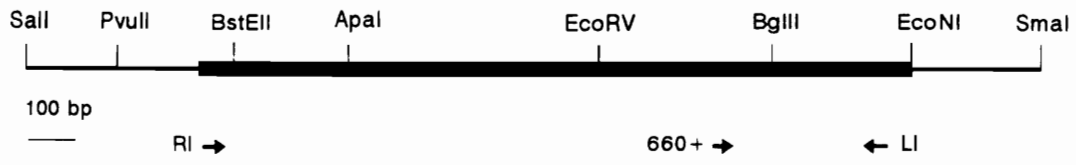

FIG. 4.1. Restriction map of IS*1141* and flanking DNA in pVT365. The IS element is marked by the filled box. Positions of primers used in PCR reactions are indicated with arrows. Primers RI and LI amplify a 1550 bp product and primers 660+ and LI amplify a 380 bp product. The 1550 bp product as well as the 1.5 kb *Bst*EII-*Eco*NI fragment were used as IS*1141* probes.

DNA from these *M. intracellulare* strains and *M. scrofulaceum* strain TMC1323^T were tested for strength of radioactive signal produced when hybridized with radiolabeled IS*1141* DNA. The strains differ in the number and position of IS*1141* copies (see Fig. 4.2 legend), so the detection limit could be determined. For each method, 1 ml of each MGE culture with turbidity equivalent to 0.5 to 1 McFarland standard was centrifuged (8,000 x g for 5 min at 4° C) in a 2 ml screw-cap microcentrifuge tube. The cell pellets were suspended in 1 ml of TE (Sambrook *et al.*, 1989) before further processing. To determine if method of growth influenced detection, strain Va14(T) colonies from MGE agar plates were added to 4 ml of TE until turbidity matched the McFarland standard and 1 ml aliquots were processed in parallel with the broth cultures.

For the chloroform glass-bead method, 300 µl chloroform and 1 g of 0.1 mm glass beads were added to the cells suspended in TE in a 2 ml microcentrifuge tube, the tube was agitated for 3 min in a Mini Bead-beater (Biospec Products, Bartlesville, OK), and debris removed by centrifugation (5,000 x g for 5 min at 4° C). In the TE glass-bead method, an additional 300 µl of TE and 1 g of beads were added to the tubes and the samples were processed in the Mini-Bead beater and centrifuged as described above. For the NaOH glass-bead method, the cells were mixed with 300 µl of 2.0 M NaOH and 1 g of beads, broken in the Mini Bead-beater and centrifuged. In the boiling method, the cells were boiled in 1.3 ml of 10 mM Tris HCl (pH 8.0) containing 1 mM EDTA and 0.4 M NaOH for 10 min and centrifuged (10,000 x g for 5 min at 4° C) as described by the manufacturers instructions with the Bio-Dot vacuum blotter (Bio-Rad Laboratories, Inc.; Richmond, CA).

The prepared DNAs were vacuum-blotted onto Zeta-probe membrane (Bio-Rad

Laboratories, Inc.; Richmond, CA) that had been pre-wet with 2X SSC (0.3 M NaCl, 30 mM trisodium citrate pH 7.0; Sambrook *et al.*, 1989) and assembled in the Bio-Dot apparatus. For the three glass-bead methods, 300 µl of each DNA sample was added to the Bio-Dot wells, while for the boiling method, 500 µl of the DNAs were added to the Bio-Dot wells. Vacuum was applied to the apparatus until the wells were empty of liquid and 500 µl of 0.4 M NaOH was added and immediately removed by vacuum. The apparatus was disassembled and the membrane was washed in 2X SSC, air dried, and hybridized as described below.

Two hybridization solutions were tested with the cell disruption methods described above. The first consisted of 50% formamide, 4X SSPE (0.72 M NaCl, 40 mM Na_2HPO_4, 1 mM EDTA, pH 7.4), 1% sodium dodecyl sulfate (SDS) and 0.5% BLOTTO (Sambrook *et al.*, 1989). In the second, the concentration of formamide was reduced to 45%. DNA-coated membranes were incubated with agitation in one of these two solutions with 5 x 10^5 cpm/ml IS*1141* probe (Fig. 4.1) at 50° C for 16 h and washed as directed by the Zeta-probe manufacturer with a final wash in 0.1 SSC (15 mM NaCl, 1.5 mM trisodium citrate), 1% SDS for 20 min at 50° C.

Species identification of isolates. Mycobacterial isolates to be identified as well as the standard Trudeau Memorial Collection type strains (*M. avium* strain TMC724^T, *M. intracellulare* strain TMC1406^T, and *M. scrofulaceum* strain TMC1323^T) were disrupted by the NaOH glass-bead method and vacuum-blotted on to Zeta-probe membrane with the Bio-Dot (described above). One membrane was prepared for each MAC SNAP System (Syngene, Inc., San Diego, CA) probe (A, *M. avium*; I, *M. intracellulare;* and X, *Mycobacterium* species X) from the same disrupted culture samples. The membranes were washed in 2X SSC, air-dried,

and hybridized with the SNAP System probes using the reagents (except the centri-dot and membranes provided) and the protocol provided by the manufacturer.

Detection of IS*1141*, IS*901*, pLR7, and pVT2. Isolates to be surveyed for marker content were prepared with the NaOH glass-bead method and vacuum-blotted onto Zeta-probe membranes with the Bio-Dot. Separate membranes were prepared for each probe from the same disrupted culture samples. Known positive and negative strains for each marker was included on the membranes. The membranes were washed in 2X SSC, air-dried, and hybridized at 50° C with DNA probes in the 50% formamide solution described above.

Detection of IS*1141* by PCR. Mycobacteria suspended in TE were disrupted by agitation with 0.1 mm glass beads for 3 min in the Mini Bead-beater. After cell debris was removed by centrifugation (5,000 x g, for 5 min at 22° C), 3 µl of each cell preparation were added to two IS*1141*-fragment amplification reactions. The first reaction used primers LI (5'GTCAACCTGAATGAGGCACTGTTG) and 660(+) (5'CACGCCCACAGCTCTCCGAC GAC) to amplify a 380 bp fragment of IS*1141*. The second reaction used primers LI and RI (5' TGCCAACCTGGGTTGAGGCACCTG) to amplify a 1550 bp fragment of the 1596 bp IS*1141* (Fig. 4.1). The amplification was performed using a PTC-100 thermocycler (MJ Research, Watertown, MA) in a 30 µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 $MgCl_2$, 0.2 mM of each deoxynucleotidetriphospate (Promega, Madison, WI), 50 pmol of each oligonucleotide primer, 5% (vol/vol) dimethylsulfoxide, 2% (vol/vol) sterile glycerol, and 1 unit of *Taq* polymerase (Promega, Madison, WI). The reaction mix was overlaid with 50 µl of light mineral oil and denatured at 95° C for 5 min. The thermocycling profile consisted of 30 cycles of denaturation at 95° C for 1 min, annealing at 55° C for 1 min

and polymerization at 72° C for 3 min. Amplification products were separated by electrophoresis on a 1.2% agarose (Fisher Scientific, Pittsburgh, PA) gel in TAE buffer and stained with ethidium bromide. Amplification products were transferred to Zeta-probe membrane with 0.4 M NaOH without prior depurination by and hybridized as described below.

Plasmid DNA detection. Cultures of mycobacterial strains containing IS*1141* were grown at 37° C in 10 ml of MGE to late log-phase (12 d). After the addition of OA to bring the culture to 15% (vol/vol) OA for opaque variants and 20% (vol/vol) OA for transparent variants, the cultures were incubated for 24 to 36 h longer at 37° C with rotation. D-cycloserine and ampicillin (Crawford and Bates, 1979) were added to final concentrations of 1 and 0.1 mg/ml, respectively, and incubation continued for 24 h at 37° C with rotation. After harvest, cells were lysed and the plasmid DNA was isolated by a modification (Jucker and Falkinham, 1990) of the Kado and Liu procedure (Kado and Liu, 1981). Plasmid profiles of the Va14, Va10, and Va2 variants were examined by electrophoresis of the crude plasmid DNA through a 0.5% agarose gel (3 V/cm). The gel was stained with ethidium bromide (0.5 µg/ml) and photographed before Southern blotting.

Genomic DNA isolation. DNA was isolated from 20 ml cultures of strains containing IS*1141*. The cultures were centrifuged (5,000 x g for 15 min at room temperature) and the cell pellets suspended in 0.7 ml of TE. The cell suspensions were transferred to 2 ml screw-cap microcentrifuge tubes, 1 g of 0.1 mm beads was added and the cells were disrupted for 1 min in the Mini Bead-beater. The cell suspension was treated with 50 µg proteinase K per ml at 65° C for 20 min, NaCl was added to the solution to a final concentration of 0.5 M, mixed, and 80 µl of a 10% (wt/vol) hexadecyltrimethlyammonium bromide, 0.7 M NaCl

solution (Wilson, 1987) was added. Incubation at 65° C was continued for 10 additional min before the cell lysate was cooled to room temperature. An equal volume of chloroform-isoamyl alcohol (24:1) was added, mixed and centrifuged (5,000 x g for 5 min at 22° C) to separate the aqueous and organic phases. The DNA-containing aqueous phase was transferred to a new microcentrifuge tube and the DNA was precipitated with 2 volumes of 95% (vol/vol) ethanol. The microcentrifuge tube was inverted gently to mix the aqueous and alcohol phases and the DNA clot that formed was removed on a glass rod, washed with 70% (vol/vol) ethanol, and after drying, dissolved in TE. Restriction endonuclease digests were prepared by standard protocols (Sambrook *et al.*, 1989) and fragments were separated by electrophoresis through 1% agarose gels.

Genomic and plasmid DNA hybridization. After electrophoresis, the DNA in agarose gels was depurinated in 0.25 M HCl for 10 min, denatured in 0.4 M NaOH for 30 min before alkaline transfer to Zeta-Probe membranes according to manufacturer's instructions (Bio-Rad Laboratories, Inc., Richmond, CA). DNA probes were hybridized with genomic and total plasmid DNA at 50° C (in 50% formamide, 4X SSPE, 1% SDS and 0.5% BLOTTO) and washed according to manufacturer's directions. Autoradiographs of air-dried membranes were exposed at room temperature using Kodak X-Omat film.

RESULTS

Dot blot optimization. With each DNA preparation method, differences in image intensity correlated with the copy number of IS*1141* in the strains (Fig. 4.2). Strain Va14(T) has two chromosomal copies and one plasmid copy of IS*1141* (column A) and strain Va14(O) has three chromosomal and two plasmid copies of IS*1141* (column B). Strain Va10-63 (column D) has three chromosomal copies of IS*1141*. *M. scrofulaceum* strain TMC1323 (column E) lacks IS*1141*. No difference in signal intensity was observed with Va14(T) cells grown in MGE broth (column A) or on MGE agar (column C). The chloroform glass-bead, TE glass-bead, and NaOH glass-bead procedures each released sufficient DNA to yield an intense, useable positive signal (rows 1-3). The boiling method yielded either insufficient DNA or poor DNA preparations producing weak positive signals which made differentiating between strains with and without IS*1141* difficult (row 4). A 5% difference in formamide concentration did not appear to significantly affect the image density of positive verses negative signals. Additional experiments showed that the addition of 0.4 M NaOH to the dot blot wells following DNA collection was unnecessary in the NaOH glass-bead procedure (data not shown). Due to ease of preparation, the NaOH glass-bead procedure was used in all subsequent experiments.

Dot blot species identification. The Virginia clinical non-AIDS pulmonary isolates (Table 4.1) were classified using the SNAP probes and the NaOH glass-bead dot blot method to prepare the culture samples. Nine *M. avium*, 20 *M. intracellulare*, 1 *Mycobacterium* species X, and three isolates which did not react with any of the SNAP MAC DNA-probes were

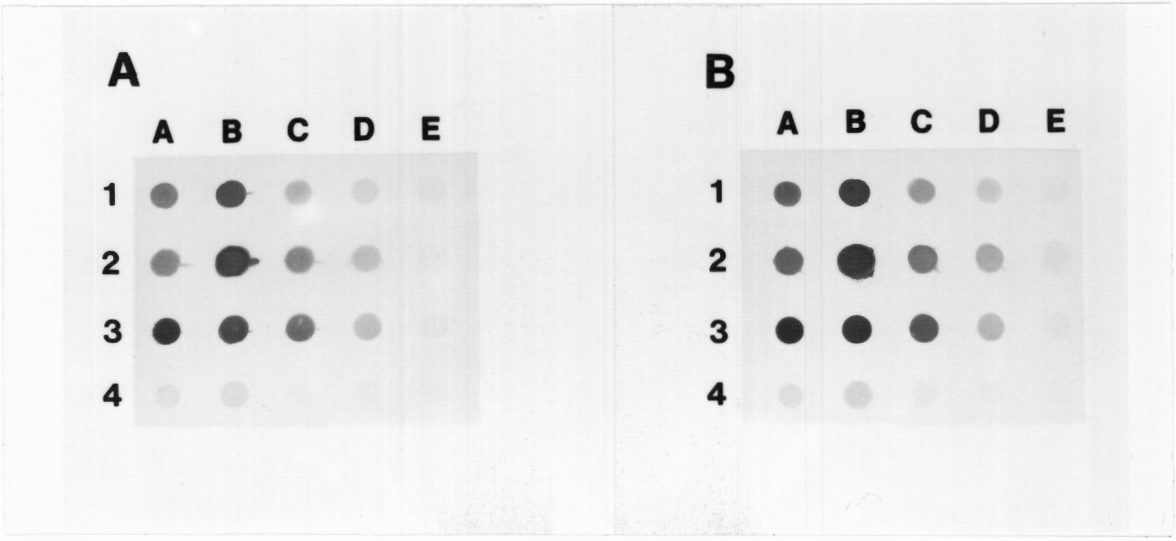

FIG. 4.2. Dot-blot optimization experiment for detection of IS*1141*. Panel A shows hybridization signal under stringent conditions. Panel B shows hybridization signal under less stringent conditions. Rows 1 through 4 correspond to the four DNA isolation methods described in Material and Methods. Row 1, chloroform glass-bead method; row 2, TE glass-bead method; row 3, NaOH glass-bead method; and row 4, boiling method. The columns contain DNA from a single strain. Column A, *M. intracellulare* strain Va14(T) grown in MGE [3 copies IS*1141*], column B, Va14(O) grown in MGE [5 copies], column C, Va14(T) grown on MGE agar [3 copies], column D, Va10-63 grown in MGE [3 copies], and column E, *M. scrofulaceum* strain TMC1323^T grown in MGE [no copies].

identified. The failure of an MAC isolate to react with the MAC probes is not unexpected since there appear to be MAC species in addition to *M. avium*, *M. intracellulare*, and *Mycobacterium* species X (Wayne *et al.*, 1991).

The Virginia aerosol and water isolates (Table 4.2) were identified by dot-blot using the SNAP DNA probes. Twelve *M. avium*, four *M. intracellulare*, and nine *Mycobacterium* species X were identified. Six isolates failed to hybridize with any of the probes. The SNAP probes and the described procedure correctly identified strain TMC724^T as *M. avium* and strain TMC1406^T as *M. intracellulare*. *M. scrofulaceum* strain TMC1323^T did not hybridize with any of the SNAP MAC probes.

Dot blot detection of IS*1141*, IS*901*, pLR7, and pVT2. The presence of IS*1141*, IS*901*, pLR7, and pVT2 in the MAC isolates, as judged by dot blot hybridization, is summarized in Tables 4.1 and 4.2. In addition to *M. intracellulare* strain Va14, two pulmonary *M. intracellulare* isolates, Va2 and Va10, hybridized to the IS*1141* probe (Table 4.1). Of the four environmental *M. intracellulare* isolates, only isolate 4S hybridized to the IS*1141* probe (Table 4.2). None of the *M. avium*, *M. scrofulaceum*, *Mycobacterium* species X, or MAC isolates appeared to hybridize significantly with IS*1141*.

Three environmental isolates, *M. avium* 2S, *Mycobacterium* species X JR1388, and *Mycobacterium* species X W33, hybridized to the IS*901* probe (Table 4.2). No pulmonary isolates hybridized with IS*901* (Table 4.1). DNA hybridizing to pLR7 was detected in three clinical *M. avium* isolates and three *M. avium* aerosol isolates (Tables 4.1 and 4.2). pLR7-hybridizing DNA was not detected in species other than *M. avium*.

Among the clinical isolates, three *M. avium* (Va3, Va5, and Va23) and two *M.*

intracellulare (Va2 and Va10) contained DNA which hybridized with the pVT2 plasmid (Table 4.1). Among the environmental isolates, *M. avium* 5S and *M. intracellulare* 4S both hybridized with pVT2 DNA (Table 4.2). Those isolates hybridizing to pVT2 contained 12 to 15 kb plasmids (Meissner and Falkinham, 1986). Each of the *M. intracellulare* isolates that contained pVT-hybridizing DNA also contained IS*1141*.

PCR amplification of IS*1141*. Due to the presence of several weak positive signals on the IS*1141*-probed dot blot (data not shown) and the lack of a isolate known to contain a single copy of IS*1141*, two PCR assays designed to detect IS*1141* were performed. The PCR annealing temperature was reduced (from 58° to 55° C) to increase the chances of amplification of any possible IS*1141*-related sequences. The amplification products from 16 clinical and nine environmental MAC isolates as well as the type strains of *M. avium, M. intracellulare* and *M. scrofulaceum* were separated by agarose gel electrophoresis and photographed (Fig. 4.3A) before Southern blotting. Many DNA amplification products were produced (Fig. 4.3A, row 1), but only lanes 2, 7, 10, 17, 21, 23, and 27 had a 380 bp band which was the expected product of primers LI and 660+. Further, DNA bands in lanes 2, 7, 10, 21, and 27 were similar in size to the 1550 bp amplification product of IS*1141* by primers LI and RI (Fig. 4.3A, row 2). These lanes correspond to isolates Va2, Va10, Va14, 4S, and W33. Upon Southern blotting and hybridization of these PCR products with an IS*1141* probe, the 380 bp and 1550 bp products of isolates Va2, Va10, Va14, and 4S hybridized with the IS*1141* probe (Fig. 4.3B). The amplified product of *M.* species X isolate W33 did not hybridize to the IS*1141* probe (Fig. 4.3B).

FIG. 4.3 legend. (A) Agarose gel of PCR products amplified with IS*1141* internal primers from MAC target DNA and (B) hybridized with an IS*1141* probe. Products in row 1 were amplified with primers LI and 660 which amplify a 380 bp product (arrow) from target DNA containing IS*1141*. Products in row 2 were amplified with primers LI and RI which amplify a 1550 bp IS*1141* fragment (arrow). Reactions containing DNA from the same isolate were loaded in the same lane in rows 1 and 2. The *M. avium* complex isolates amplified with IS*1141* primers were: lane 1, Va1; lane 2, Va2; lane 3, Va3; lane 4, Va5; lane 5, Va6; lane 6, Va8; lane 7, Va10; lane 8, Va12; lane 9, Va13; lane 10, Va14; lane 11, Va15; lane 12, Va16; lane 13, Va26; lane 14, Va27; lane 15, Va32; lane 16, Va33; lane 17, *M. avium* strain TMC724; lane 18, *M. scrofulaceum* strain TMC1323; lane 19, *M. intracellulare* strain TMC1406; lane 20, 2S; lane 21, 4S; lane 22, 9S; lane 23, 14S; lane 24, JR584; lane 25, JR696; lane 26, JR959; lane 27, W33; lane 28, W277; lane 29, W280; lane 30, no DNA. In row 1 a 100 bp marker and in row 2 a 100 bp and 1 kb marker (Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD) were included. (B) Autoradiograph of Southern blot of the gel in Fig 4.3A hybridized with a IS*1141* probe made by random priming.

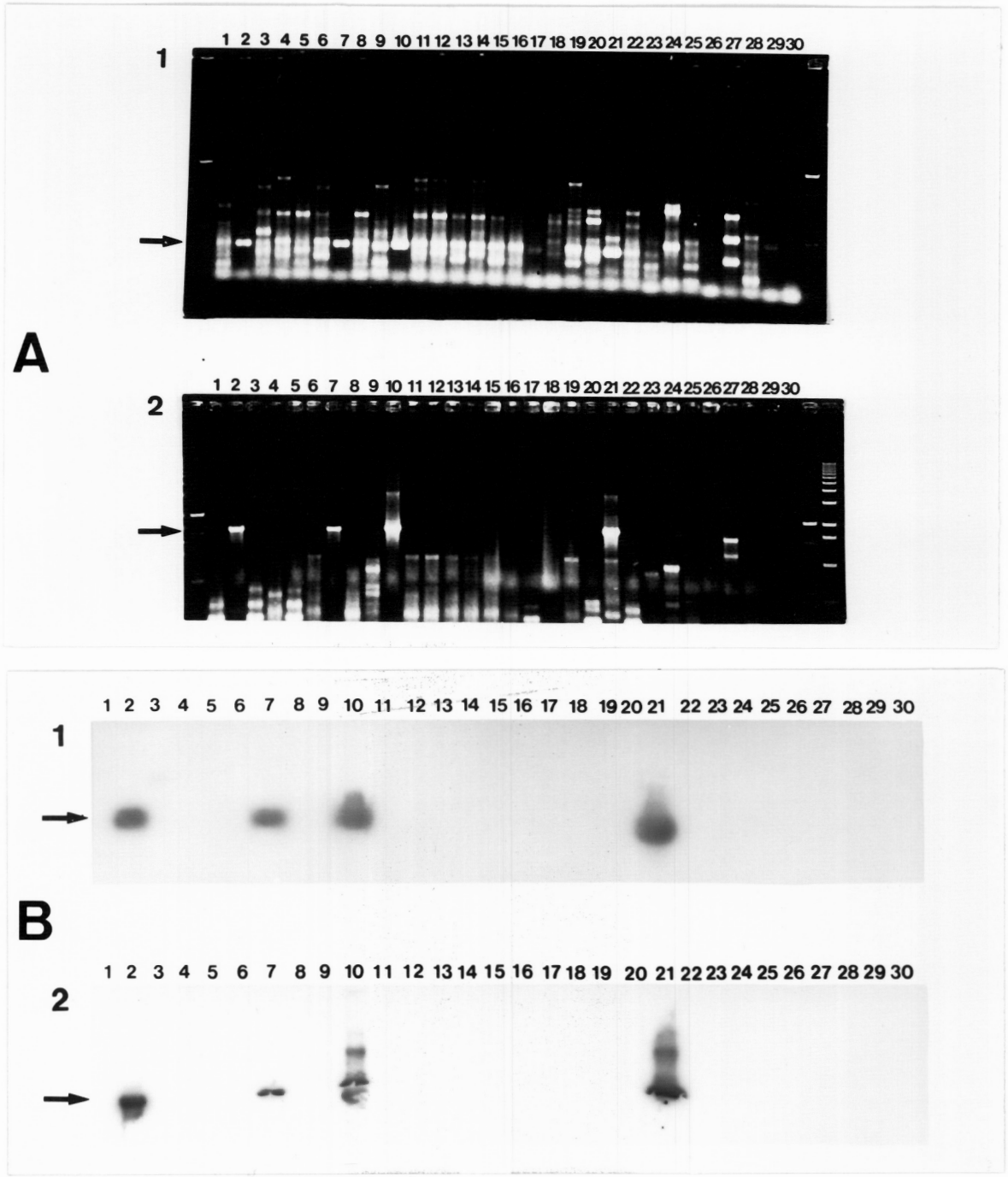

Fig. 4.3. (A) PCR products amplified with IS*1141* internal primers from *M. avium* complex target DNA and (B) hybridized with an IS*1141* probe.

Presence of IS*1141* in plasmids. *M. intracellulare* isolate 4S plasmids were not examined due to the rough colony morphology of the isolate which makes cell lysis and plasmid detection very difficult. Plasmid DNA was released from pairs of Va14, Va10 and Va2 strains with different plasmid profiles (Erardi *et al.*, 1985) and separated by agarose electrophoresis (Fig. 4.4A). On the gel, the parent strain of each pair is followed by the derivative containing a plasmid approximately 5 kb smaller than the original parent strain (Fig. 4.4A). The plasmids in the parent strain Va14(O) had apparent sizes of 240, 68, 25, and 16 kb (lane 2). The transparent derivative, Va14(0T), had plasmids of 240, 62, 25, and 16 kb (lane 3). The plasmids in parent strain Va10-33 had apparent sizes of 240, 155, 72, 25, 16, and 13.5 kb (lane 4), while the derivative, Va10-63 had plasmids of 240, 155, 66, 25, 16, and 13.5 kb (lane 5). The plasmids in the parent strain Va2-11 had apparent sizes of 135, 66, 32, 25, 17, and 13.5 kb (lane 6), and the derivative, Va2-12, had plasmids of 135, 66, 27, 25, 17, and 13.5 kb (lane 7). The plasmid DNA was transferred to Zeta-probe membrane and hybridized with the IS*1141* probe. *M. intracellulare* strains Va14(O), Va14(OT), Va10-33 and Va2-11 each had plasmids which hybridized to IS*1141*. In each strain, the parental plasmid observed to change size hybridized to IS*1141* and the presumed smaller plasmid derivative had lost the DNA which hybridized to IS*1141*. In strain Va14 both the 68 and the 16 kb plasmids hybridized to IS*1141*. The copy of IS*1141* in the 16 kb plasmid was partially sequenced (Appendix B) and in the regions sequenced was identical to the IS*1141* sequence from the 68 kb plasmid (data not shown). The 16 kb plasmid has not been observed to lose its one copy of IS*1141*, but derivatives have been isolated where the 16 kb plasmid had two copies of IS*1141* (Fig. 3.4 and 3.11).

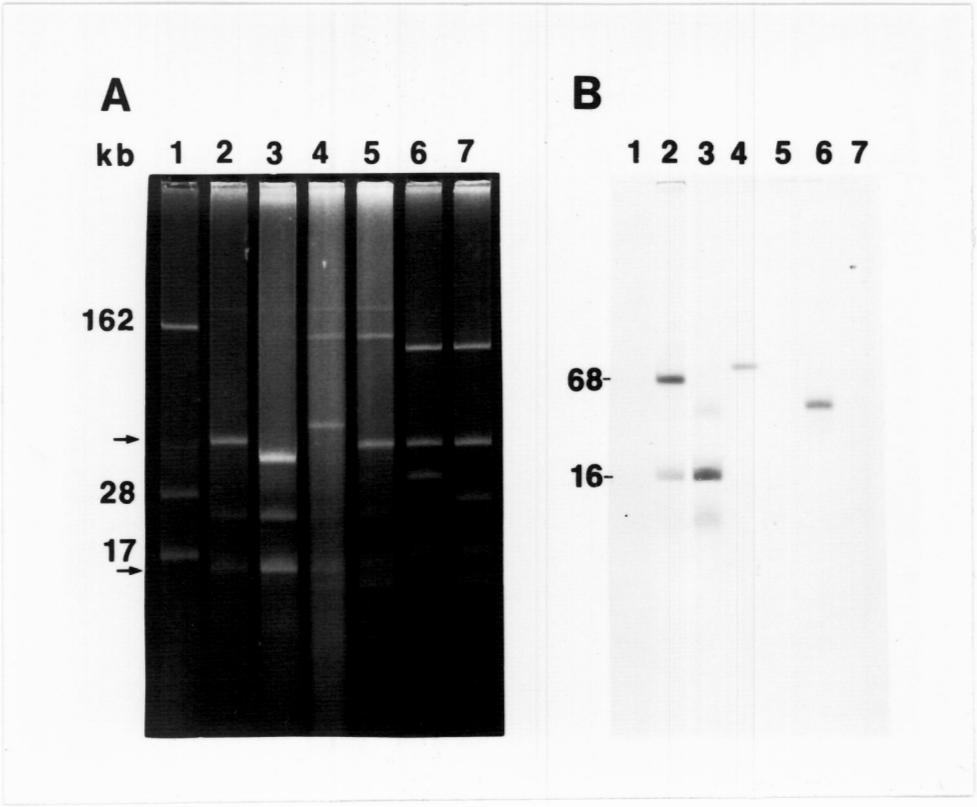

FIG. 4.4. (A) Agarose gel electrophoresis and (B) Southern hybridization of undigested plasmid DNA prepared from *M. intracellulare* strains with an IS*1141* probe. Lane 1, *M. avium* strain LR25 plasmids (162, 28 and 17 kb) were used as molecular mass markers (Crawford *et al.*, 1981). Lane 2, *M. intracellulare* strain Va14-83(O); lane 3, Va14-83(OT); lane 4, Va10-33; lane 5, Va10-63; lane 6, Va2-11; lane 7, Va2-12. (B) Autoradiograph of a Southern blot of gel (A), which was hybridized with the radiolabeled 1.5 kb *Apa*I-*Sma*I fragment of IS*1141* (pVT328, Fig. 3.3). The 68 and 16 kb plasmids of Va14-83(O) which hybridize with IS*1141* are marked.

The 68 kb plasmid (pVT203) of strain Va14(O) contained a 1.8 kb region which appeared to be duplicated on either side of the IS*1141* insertion (Fig. 3.3). The 62 kb plasmid in strain Va14(OT) has only one copy of this 1.8 kb region (Fig. 3.3). To determine if this flanking DNA was present in the plasmids of strains Va10 and Va2, a Southern blot of the plasmids was hybridized with a 0.5 kb *Bam*HI-*Pst*I fragment from the duplicated region of pVT203 (Fig. 3.3). In each strain, both the parental plasmid and the smaller plasmid hybridized to the fragment of the duplicated region from the 68 kb plasmid of strain Va14 (Fig. 4.5).

Presence of IS*1141* in genomic DNA. Two copies of IS*1141* were present in the chromosome of transparent variants of strain Va14 and three in opaque variants of IS1141 (Fig. 3.11). To determine if IS*1141* was present in the chromosome of *M. intracellulare* strains Va10, Va2, and 4S, genomic DNA from each strain was digested with the restriction endonuclease *Sal*I, the fragments separated by agarose gel electrophoresis, and a Southern blot of the DNA was hybridized with the IS*1141* probe (Fig. 4.6).

The inclusion of digested Va14(O) total plasmid DNA (lane 1) allowed the identification of the 3.7 kb band from the 68 kb plasmid (pVT203) and the 2.4 kb band from the 16 kb plasmid (Fig. 4.6). Va14(O) had three chromosomal copies of IS*1141* (Fig. 4.6, lane 2) and Va14(OT) had two chromosomal copies (Fig. 4.6, lane 3) as was described earlier (Fig. 3.11). Strain Va10-33 had three chromosomal copies of IS*1141* and one (3.0 kb band) from the 72 kb plasmid (Fig. 4.6, lane 4). Strain Va10-63 (lane 5) contained IS*1141* on the same sized chromosomal DNA fragments as strain Va10-33. Strain Va2-11 had one plasmid copy of IS*1141* and one chromosomal copy (lane 6). The plasmid DNA fragment from

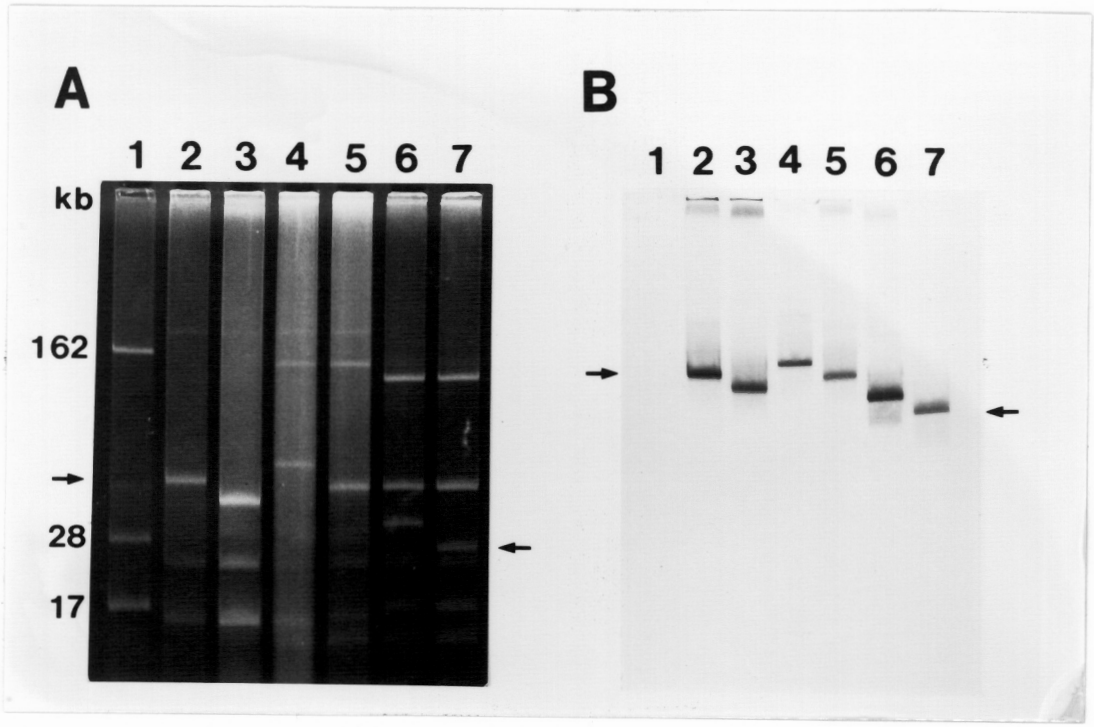

FIG. 4.5. (A) Agarose gel electrophoresis and (B) Southern hybridization of undigested plasmid DNA prepared from *M. intracellulare* strains with a pVT203 probe. Lane 1, *M. avium* strain LR25 plasmids (162, 28 and 17 kb) were used as molecular mass markers (Crawford *et al.*, 1979). Lane 2, *M. intracellulare* strain Va14-83(O); lane 3, Va14-83(OT); lane 4, Va10-33; lane 5, Va10-69; lane 6, Va2-11; lane 7, Va2-12. (B) Autoradiograph of a Southern blot of gel (A), which was hybridized with the radiolabeled 0.5 kb *Bam*HI-*Pst*I fragment of pVT328, shown in Fig. 3.3. The 68 kb plasmid of Va14-83(O) and the 27 kb plasmid of Va2-12 which hybridized to the probe are marked with arrows.

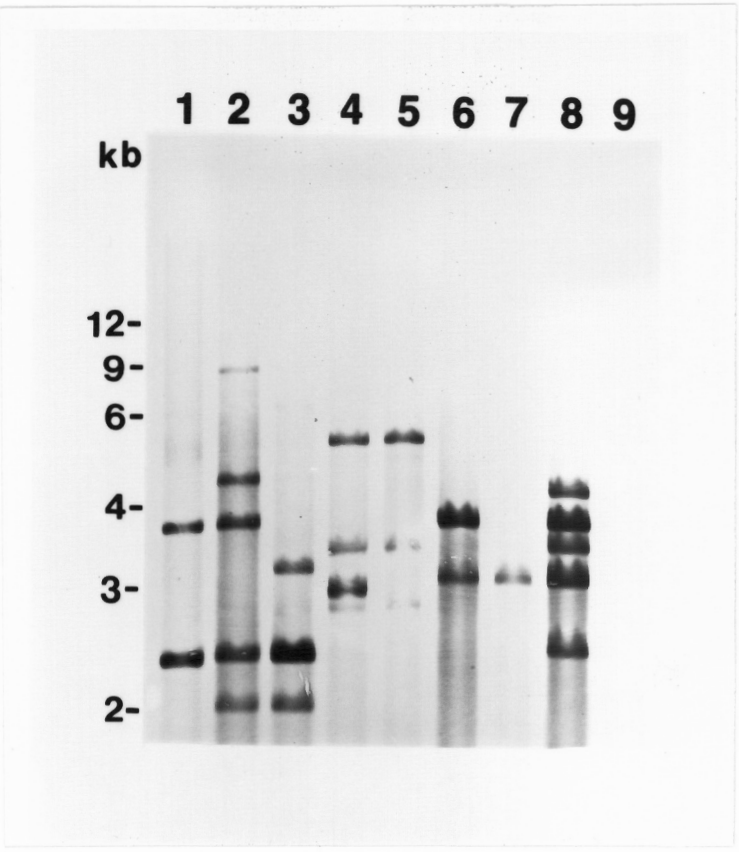

FIG. 4.6. *M. intracellulare Sal*I digested genomic DNA hybridized with the IS*1141* probe. Lane 1, Va14(O) plasmid DNA; lane 2, Va14(O); lane 3, Va14(OT); lane 4, Va10-33; lane 5, Va10-63; lane 6, Va2-11; lane 7, Va2-12; lane 8, 4S; lane 9, TMC1406^T DNA. The molecular mass markers (kb) at the left side represent the 1 kb ladder (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD).Va2-11 was the same size as the plasmid fragment from the 68 kb plasmid of strain Va14(O). Strain Va2-12 contained only one chromosome copy of IS*1141* (lane 7) and it was on the same size fragment as was seen in Va2-11.

Isolate 4S had five bands which hybridized with IS*1141* (Fig. 4.6, lane 8). The differences in intensity of plasmid and chromosomal bands and the sizes of the DNA fragments hybridizing with IS*1141* suggest that isolate 4S has two copies of IS*1141* (3.7 and 2.4 kb bands) in plasmids (Meissner and Falkinham, 1986) and three in the chromosome (Fig. 4.6). The *M. intracellulare type* strain TMC1406 lacked IS*1141* (lane 9).

Restriction endonuclease digestions of DNA from these IS*1141*-containing strains with *Bam*HI and *Eco*RV and hybridization with the IS*1141* probe showed similar IS1141 copy numbers and genome distribution of IS*1141* (data not shown) as described for *Sal*I DNA digestion (Fig. 4.6).

DISCUSSION

The objective of this study was to identify the distribution of IS*1141* in MAC isolates. To accomplish this, several methods for releasing DNA from mycobacteria were evaluated. The method chosen for strength of signal and speed of processing involved cell breakage by glass beads with simultaneous DNA denaturation (Fig. 4.2, row 3). That NaOH glass-bead dot blot method was sufficiently sensitive to detect the presence of one chromosomal copy of IS*1141* in strain Va2-12 (data not shown). A PCR assay using pairs of IS*1141* sequences as primers amplified IS*1141* fragments (Fig. 4.3) in the same four isolates (Va14, Va10, Va2, and 4S) that hybridized in the dot blot assay (Tables 4.1 and 4.2) and in the Va14, Va10, and Va2 strains with different plasmid profiles (data not shown).

In the collection of 33 pulmonary isolates, 20 (60%) were *M. intracellulare*, nine (27%) were *M. avium*, and one (3%) was *Mycobacterium* species X (Table 4.1). IS*1141* was found in only three (15%) of the *M. intracellulare* isolates and no other MAC species. Three isolates identified as MAC by cultural and biochemical tests did not hybridize significantly with any of the SNAP system probes. Recently, in a study of 93 MAC isolates from HIV-seropositive patients, Cregan *et al.* (1992) found three isolates that were MAC probe negative. Wayne *et al.* (1991) have suggested that several uncharacterized species are part of the *M. avium* complex. These data indicate that members of these uncharacterized species may cause human infections. X probe positive isolates have also been recovered from sputa of AIDS and non-AIDS patients in San Francisco (Cregan *et al.*, 1992).

The collection of 31 environmental isolates included: 12 (39%) *M. avium*, four (13%)

M. intracellulare, nine (29%) *Mycobacterium* species X, and six (19%) isolates which did not hybridize with any SNAP probe. Isolates from each species were recovered from both the James River water and aerosol samples. One of the four (25%) *M. intracellulare* isolates, an aerosol isolate, contained IS*1141*. This isolate was recovered from stage five of the Anderson cascade sampler (Anderson, 1958) which indicates that the particle size on which the isolate entered the sampler was 1 to 3 μm. Particles of this size can be deposited in the lung alveolar sacs should they be inhaled (Anderson, 1958). If a susceptible person had inhaled the particle on which isolate 4S was carried, the mycobacterium could have reached, and potentially colonized the person's alveoli. No other environmental isolate contained IS*1141* (Table 4.2).

The two isolate collections were also surveyed for IS*901*, pLR7, and pVT2 content. The two (22%) environmental *Mycobacterium* species X isolates hybridizing with IS*901* are the first reported. IS*901*-containing *M. avium* isolates are mostly animal isolates (Kunze *et al.*, 1992) including the *M. avium* subsp. *silvaticum* (Moss *et al.*, 1992). Possible animal-waste contamination of the James River cannot be ruled out as the source of the three IS*901*-containing isolates.

M. avium isolates with plasmids were likely to contain pLR7. Three of the four (75%) non-AIDS pulmonary isolates with plasmids hybridized with pLR7 and three of seven (43%) environmental isolates with plasmids hybridized with pLR7. Only *M. avium* SNAP probe positive isolates hybridized with pLR7 (Table 4.1 and 4.2). Recent SNAP probe testing of the isolates characterized by Jucker and Falkinham (1990) indicated that only the *M. avium* isolates contained pLR7-related plasmids (data not shown).

M. avium and *M. intracellulare* isolates from both collections hybridized with the

pVT2 plasmid probe. Restriction endonuclease mapping of representative pVT2-related plasmids showed that there were large conserved regions in pVT2-related plasmids (Jucker, 1992) isolated from either *M. avium* or *M. intracellulare*. In the collections surveyed, all *M. intracellulare* isolates that hybridized with pVT2 contained IS*1141* (Table 4.1 and 4.2), but IS*1141* was not located on the plasmids in the 12 to 15.3 kb size-range (Fig. 4.4) of pVT2-related plasmids (Jucker and Falkinham, 1990). Strain Va14 did not contain a plasmid in the size-range of pVT2, but the strain does contain IS*1141* (Fig. 4.4). Any relationship between IS*1141* content and pVT2 content of *M. intracellulare* isolates remains to be examined.

The *M. intracellulare* strains Va14, Va10, Va2, and 4S were examined to determine the location and copy number of IS*1141*. Southern hybridization of the plasmids of Va14, Va10, and Va2 strains with different plasmid profiles (Erardi *et al.*, 1985; Via and Falkinham, 1991a) demonstrated IS*1141*'s presence in both small (16 kb) and large (32 to 72 kb) plasmids (Fig. 4.4). The large plasmids of Va10 and Va2 strains also hybridized to a DNA fragment adjacent to the IS*1141* insertion in the 68 kb plasmid (pVT203) of Va14 (Fig. 4.5). This may indicate a conserved region containing a IS*1141* target sequence shared by the plasmids. Alternately, a larger mobile DNA fragment carrying the IS*1141* insertion sequence may have integrated into these plasmids at some point in the past.

Genomic digests show that Va14(O) and Va14(OT) colonial variants had different chromosomal IS*1141* RFLP patterns (Fig. 4.6 and 3.11), but any relationship between IS*1141* RFLP patterns in isolates Va10, Va2, and 4S and these isolates' colonial morphology remains to be determined. Each of the isolates with IS*1141* had a unique RFLP pattern.

It was our hope that IS*1141* would, like IS*6110* from *M. tuberculosis*, be useful as an

epidemiologic marker for MAC organisms. Although the isolates containing IS*1141* had different IS*1141* RFLP patterns, IS*1141* was present in only 17% of the *M. intracellulare* isolates. The 64 isolates surveyed were isolated from patients and environmental samples in the James River basin. It remains to be seen if IS*1141* is present in localities other than the James River basin. Consequently, IS*1141* appears to be of limited use in epidemiologic studies. Its possible use in transposon mutagenesis of MAC remains to be investigated.

CHAPTER V

Summary

The main objective of this study was to investigate the mechanism of colonial variation in *Mycobacterium avium* complex (MAC). Most published studies have been limited to characterization of the transparent (T) and opaque (O) variants and the authors have only suggested mechanisms. Previously suggested mechanisms for colonial variation in MAC organisms include mutation (McCarthy, 1970; Kajioka and Hui, 1978) and lysogeny (Jones and White, 1967). Mutation is unlikely as a mechanism because the rates of colonial variation are far higher than mutation rates (McCarthy, 1970; Woodley and David, 1976) and the colony types are interconvertible (Woodley and David, 1976). Because the lysogeny model described reversion from rough to smooth coincident with phage lysogeny, and not O to T interconversion, it has not been explored further. Mizuguchi *et al.* (1981) described the concurrent loss of a 3 kD plasmid and transition from T to O morphology in a *M. intracellulare* strain grown with Mitomycin C. Because of the strain's change in morphology and antibiotic resistance, Mizuguchi *et al.* (1981) proposed that plasmid loss was responsible for colonial variation. Plasmid loss is not a reasonable model for a reversible phenomena because reversion would not be possible unless the plasmid was reintroduced from another bacterium. Plasmid integration into the chromosome would fit the interconvertable nature of O and T variation. This mechanism has not been investigated because the strain has been lost (Mizuguchi, personal communication).

Transparent variants are antibiotic-resistant (Kuze and Uchihira, 1984), virulent in animal models (Schaefer *et al.*, 1970), and require fatty acids for growth (Kajioka and Hui, 1978), while the opaque variants are antibiotic sensitive, less virulent and able to grow without

fatty acid supplements. The simultaneous loss of pathogenicity, antibiotic resistance, and T-specific antigens upon the transition from T to O colony type (Thorel and David, 1984) has led to assumption that the expression of gene(s) encoding production of particular cell surface glycolipids is responsible for the T phenotype. Under this assumption, cells not expressing these T phenotype genes or transcribing alternate genes would form opaque colonies. The assumption that lack of T-specific gene expression causes O morphology is based on the lack of evidence of O-specific antigens (Thorel and David, 1984).

Our investigation of IS*1141* involvement in colonial variation in *M. intracellulare* strain Va14 determined that the presence of IS*1141* in the 68 and 62 kb plasmids was unrelated to colonial variation. During the investigation, the IS element was found to be present in another plasmid and the chromosome of the Va14 variants. Hybridization studies identified three chromosomal bands in O variants and two chromosomal bands in T variants of Va14. Using IS*1141* as a probe, seven Va14 T variants as well as the parental transparent had the same chromosomal restriction fragment length polymorphism (RFLP) pattern. This pattern was different from the single, unique RFLP pattern seen in seven O variants and the parental opaque. Therefore, in strain Va14, there appears to be correlation between colony morphology and IS*1141* RFLP pattern. Depending on the restriction endonuclease used, T and O variants shared either one (*Sal*I digests, Fig. 3.11) or two (*Eco*RV digests, Fig. 3.12) chromosomal bands that hybridized with IS*1141*. O variants consistently had one more copy of IS*1141* than did the T variants (Fig. 3.11).

The mechanisms of phase or antigenic variation are well characterized in only a few bacterial species. Flagellar variation in *Salmonella typhimurium* occurs through a molecular

switch which alternates between expression of two flagellin genes, H2 or H1 (Glasgow *et al.*, 1989), In this system, the promoter for the H2 flagellin gene is located on a 1 kb invertible DNA segment (Heichman and Johnson, 1990). In one orientation, the promoter directs transcription of the H2 flagellin gene and a repressor which prevents transcription of the H1 flagellin gene which is located at another site in the chromosome (Glasgow *et al.*, 1989). When the invertible region is in the opposite orientation, the H2 and repressor genes are not transcribed and H1 expression is derepressed. The Hin protein directing this site-specific inversion, is encoded in the invertible DNA region (Heichman and Johnson, 1990). The inversion of a regulatory region, as a strategy to switch between expression of two sets of genes, also occurs in *Escherichia coli* and Bacteriophages Mu and P1. If the regulatory DNA inversion mechanism causes variation in strain Va14 (Fig 3.14, model 3), it would not necessarily cause duplication or deletion of IS*1141* during inversion. Each of the O variants isolated have three copies of IS*1141* in the chromosome but each of the T variants have two copies. If the inversion mechanism is causing variation in this strain independent of IS*1141*, then O variants with two chromosomal copies and T variants with three chromosomal copies of IS*1141* should exist, but none have been isolated. It may be that the change in colony type induces replicative transposition of IS*1141* because of changes in transcription or generation of a target insertion site.

In *Neisseria gonorrhoeae*, pilin variation occurs by gene conversion of complete and partial pilin genes into a regulatory site where transcription takes place (Swanson and Koomey, 1989). There are regions of homology both outside and within the multiple, different pilin genes which allow recombination into the transcription site. (Swanson and Koomey, 1989).

Unlike the inversion system of *Salmonella*, this system produces chimeric proteins allowing greater antigenic variation (Swanson and Koomey, 1989). Gene conversion events would probably generate multiple changes in the bacterial chromosome of the Va14 (O), (TO), and (OTO) variants making it unlikely that a single RFLP patterns would be maintained among these opaque variants. There is a single IS*1141* RFLP pattern consistently seen in the transparent variants and a different but consistent pattern in opaque variants of Va14.

Colonial variation in *Campylobacter coli* is a consequence of DNA rearrangement (Guerry *et al.*, 1988). DNA rearrangement of the chromosome accompanied by replicative transposition of IS*1141* could generate the O variants isolated. Opaque variants have one more copy of IS*1141* in the chromosome than do T variants. The conflict between the number of shared IS*1141*-hybridizing bands in O and T variants following *Sal*I (Fig. 3.11) or *Eco*RV (Fig. 3.12) digestion appears to indicate that changes in the chromosomal DNA occur with variation. In *Sal*I digested chromosomal DNA, O and T variants share one IS*1141*-hybridizing band and in *Eco*RV digested DNA the O and T variants share two hybridizing bands. But, based on the number of IS*1141* copies in OT and TOT variants isolated, this model would require deletion of IS*1141* with the repair or alteration of the DNA rearrangement unless T variants with three copies of IS*1141* exist. In eight T variants studied, none have three copies of IS*1141* in the chromosome.

Precise duplication and deletion of insertion sequence-like elements in *Citrobacter* (Snelling *et al.*, 1981; Glasgow *et al.*, 1989) and *Pseudomonas* (Bartlett *et al.*, 1988) cause variation in cell surface antigens and polysaccharides. Possible affects of this mechanism include insertion of promoter sequences in frame with genes activating transcription and

insertion of DNA that interrupts or terminates transcription of active genes. Deletion of these sequences could then prevent the promoted transcription or restore transcription in genes no longer interrupted. Either of these types of insertion would change gene expression in the cell possibly affecting colonial morphology. IS3-like elements have been shown to drive transcription of adjacent genes (Galas and Chandler, 1989). A possible promoter model (Fig. 3.14, model 2a) would have the outward oriented promoters of IS*1141* provide a missing promoter for the gene(s) determining colony morphology. If we apply the promoter model to the T phenotype, T variants should have an IS*1141* copy, not present in the O variants, integrated near genes responsible for T morphology. The copy number and *Eco*RV RFLP pattern data (Fig. 3.12), for the O and T variants isolated, are in conflict with this model. Opaque variants have three copies of IS*1141* while T variants isolated have had only two chromosomal copies of IS*1141*. The *Eco*RV RFLP patterns indicate that the T variants share both of the IS*1141*-hybridizing bands they contain with the O variants (Fig. 3.12).

The promoter model could be reversed by having the IS*1141* outward promoters direct the transcription of O-specific gene(s) (Fig. 3.14, model 2b). Given the nature of the opaque variant, these genes could be expected to code for factors increasing the permeability of the cell. Porins have been recently detected in *Mycobacterium chelonae* (Trias, *et al.*, 1992); O-specific genes-products could be porins not detected by the methods used by Thorel and David (1984). A model that has IS*1141* providing a promoter for these postulated O-specific genes, would dictate that upon the excision of IS*1141* from that site, the O phenotype would revert to the T phenotype. The presence of a third chromosomal copy of IS*1141* in the O variants would be expected in this model. The rates of transposition (10^{-4} to 10^{-6}; Galas and Chandler,

1989) and perfect excision (10^{-6} to 10^{-9}; Ehrlich, 1989) of other IS elements agree with the rate of Val4 colonial variation.

A final model (Fig. 3.14, model 1) proposes that in O variants IS*1141* is integrated into the gene or operon responsible for the T phenotype, interrupting transcription. Duplication of IS*1141* in the chromosome could trigger T to O transition and precise deletion of IS1141 from a T-operon would yield variants of the T morphology (Fig. 3.14, model 1). The Val4 transparent variants have two chromosomal copies of IS*1141*, whereas the opaque strains have a third chromosomal copy of IS*1141*. The *Eco*RV RFLP patterns seen in O and T variants of Val4 support the possibility of precise duplication and deletion of IS*1141* as a mechanism for colonial variation. The rate of perfect excision for transposable elements with inverted repeats (10^{-6} to 10^{-9}; Ehrlich, 1989) matches the rate of T variant isolation (10^{-6} to 10^{-8}) from Val4 opaque variants. The T variant is the variant expected to be transcribing additional genes which could serve as a target for integration (Thorel and David, 1984) and no O-specific expression has been detected (Thorel and David, 1984). The precise duplication and/or deletion model provides the simplest explanation of the data available (Fig. 3.14, Model 1).

Our working hypothesis, consistent with all models, requires IS*1141* to have a target site in or near the chromosomal region responsible for colonial morphology in strain Val4. Investigation of the DNA flanking the IS*1141* copies should identify: (1) a gene or operon being interrupted by IS*1141*, (2) a gene or genes being transcribed from one of IS*1141*'s outward oriented promoters, or (3) a DNA region, that when used as a probe, gives different opaque and transparent RFLP patterns in *M. intracellulare* strains with T and O variants.

Although there are many questions to be addressed about control of colonial variation in *Mycobacterium avium* complex, there is now at least a marker and a defined place in the DNA to look in strain Va14.

A second outcome of this study was the identification and characterization of the transposable element IS*1141* discovered in *M. intracellulare* strain Va14. The IS elements identified in the genus *Mycobacterium* have been of two types described as typical and atypical. The *M. tuberculosis* element IS*986* (McAdam *et al.*, 1990) and the IS1081 element (Collins and Stephens, 1991; van Soolingen *et al.*, 1992) from *M. bovis* are typical insertion sequences containing terminal inverted repeats flanked by short direct repeats. The elements are present in the genome of their hosts in multiple copies which vary in position from strain to strain. The IS*986* large ORF has some similarity to the putative transposase of the IS*3* element family (McAdam *et al.*, 1990). The other mycobacterial group of IS elements are atypical in nature lacking terminal inverted repeats and direct repeats of the insertion site. These elements, IS*900* (Green *et al.*, 1989), IS*901* (Kunze *et al.*, 1991) and IS*902* (Moss *et al.*, 1992), do appear to have rather specific target insertion sites which are regenerated once the IS element is inserted (Kunze *et al.*, 1991).

IS*1141* is 1596 bp in length (Fig. 3.7) with 23 bp terminal inverted repeats. Data base searches and a multiple sequence alignment (Fig. 3.10) indicate that IS*1141* ORFb has significant similarity to the IS*3* family transposases and IS*986*, an IS*3*-like element (McAdam *et al.*, 1990), from *M. tuberculosis*. Nucleotide and putative amino-acid sequence similarity between the other *M. avium* complex (IS*901* and IS*902*) IS elements is insignificant, but IS*1141* does have a target site (Fig. 3.8) similar to that of IS*900* (Green *et al.*, 1989) and

IS*901* (Kunze *et al.*, 1990) of *M. avium* complex as well as IS116 from *Streptomyces clavigerus* (Leskiw *et al.*, 1990). Although the ends of IS*1141* somewhat resemble the ends of these atypical elements, it is not clear whether the IS*900*-like ends or the IS*3*-like inverted repeats of IS*1141* or both are involved in transposition. Because IS*1141* transposition can be detected and measured, a study of the mechanism of transposition in mycobacteria can be initiated.

In reviewing the structure of IS*3*, IS*911*, and IS*986*, each has a poor Shine-Dalgarno sequence proceeding ORFb (Prere, *et al.*, 1990; McAdam *et al.*, 1990) similar to IS*1141*. IS*1141* ORFc overlaps ORFb in a -1 orientation like the overlap seen in IS*3*. This arrangement suggests ribosomal read-through or re-initiation as a possible mechanism for translation of ORFb (Prere *et al.*, 1990; McAdam *et al.*, 1990). The structure of IS*986* is similar in that two small ORFs proceed ORFb but the termination of ORFa2 and the beginning of ORFb overlap by approximately 100 bp. McAdam *et al.*, (1990) have suggested translational frameshifting as the mechanism of translation of IS*986* ORFb. If IS*1141* produces a fused ORFc-ORFb protein product, it is likely to occur by ribosome read-through.

Most of the IS*3* family elements have an ORF on the reverse strand in the transposase coding region (Galas and Chandler, 1989; Prere *et al.*, 1990). IS*1141* has two small ORFs in that region which are likely to be transcribed. Several IS elements, including IS*3*, have been observed to activate transcription of genes downstream from their insertion site due to either outward orientated promoters or the formation of new promoters using an outward orientated (-)35 promoter region (Galas and Chandler, 1989). IS*1141* has sequences similar to outward oriented promoters located at both ends of the element (Fig. 3.7) as well as outward oriented (-

)35 promoter regions. Due to the similarities between IS*1141* and the IS*3*-like elements IS*911* and IS*986*, we propose IS*1141* to be a member of the IS*3* family.

The last outcome of this study was an understanding of the distribution of IS*1141* in MAC organisms. The isolates surveyed were isolated from non-AIDS patients (from which strain Va14 is a member) and environmental samples in the James river basin. In the collection of 33 pulmonary isolates, 20 (60%) were *M. intracellulare*, nine (27%) were *M. avium*, and one (3%) was *Mycobacterium* species X (Table 4.1). IS*1141* was found in only three (15%) of the 20 *M. intracellulare* isolates and no other MAC species. The collection of 31 environmental isolates included: 12 (39%) *M. avium*, four (13%) *M. intracellulare*, nine (29%) *Mycobacterium* species X, and six (19%) strains which did not hybridize with any SNAP probe. One of the four (25%) *M. intracellulare* isolates, an aerosol isolate, contained IS*1141*. This isolate was recovered from stage five of the Anderson cascade sampler (Anderson, 1958) which indicates that the particles could potentially be deposited in the lung alveoli should they be inhaled (Anderson, 1958).

Southern hybridization of the plasmids of Va14, Va10, and Va2 strains with different plasmid profiles (Erardi *et al.*, 1985; Via and Falkinham, 1991a) demonstrated IS*1141*'s presence in both small (16 kb) and large (32 to 72 kb) plasmids (Fig. 4.4). The large plasmids of Va10 and Va2 strains also hybridized to a DNA fragment adjacent to the IS*1141* insertion in the 68 kb plasmid (pVT203) of Va14 (Fig. 4.5). This may indicate a conserved region containing a IS*1141* target sequence shared by the plasmids. Alternately, a larger mobile DNA fragment carrying the IS*1141* insertion sequence may have integrated into these plasmids at some point in the past.

In the collections surveyed, all *M. intracellulare* isolates that hybridized with pVT2 contained IS*1141* (Table 4.1 and 4.2), but IS*1141* was not located on the plasmids in the 12 to 15.3 kb size-range (Fig. 4.4) of pVT2-related plasmids (Jucker and Falkinham, 1990). Strain Va14 did not contain a plasmid in the size-range of pVT2, but the strain does contain IS*1141* (Fig. 4.4). Any relationship between IS*1141* content and pVT2 content of *M. intracellulare* isolates remains to be examined. If one assumes that mobile genetic elements are transferred by mycobacteriophage or unidentified mycobacterial conjugative plasmids, isolates from a population containing as IS element would be more likely to also have the IS element than isolates from other populations without the element. It remains to be seen if IS1141 is present in *M. avium* complex isolates recovered from localities other than the James river basin. Due to the apparent host sharing between pVT2 and IS*1141*, other *M. intracellulare* strains containing pVT2-related plasmids should be surveyed for IS*1141* content.

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APPENDICES

LV# numbers	VA14 name	Parent strain	Morphology	Plasmid content of strain 240	68	62	25	18	16	Profile changed	RFLP Pattern
LV1	VA14-83(O)	VA14	opaque	X	X		X		X		O
LV2	VA14-89(T)	VA14-O	transparent	X		X	X		X	Y	T
LV11	VA14-83(OT)	LV1	transparent	X		X	X		X	Y	T
LV21	VA14-89(TO)	LV2	opaque	X	X		X		X	Y	O
LV12	83(OT)G	LV1	transparent	X	X		X		X	N	ND
LV13	83(OT)H	LV1	transparent	X	X		X		X	N	ND
LV14	83(OT)J	LV1	transparent	X	X		X		X	N	ND
LV15	83(OTO)F	LV11	opaque	X	X		X		X	Y	O
LV16	83(OT2)	LV11	transparent	X		X	X		X		ND
LV17	83(OT2O1)	LV16	opaque	X	X		X		X	Y	O
LV18	83(OT3)	LV11	transparent	X		X	X		X		ND
LV19	83(OT3O1)	LV18	opaque	X		X	X		X	N	O*
LV110	83(OT4)	LV11	transparent	X		X	X		X		ND
LV111	83(OT4O1)	LV110	rough	X		X	X		X	N	T
LV112	83(OT4O2)	LV110	rough	X		X	X	X		Y	T
LV113	83(OT6)	LV11	transparent	X		X	X		X		ND
LV114	83(OT6O1)	LV114	opaque	X	X		X		X	Y	O
LV115	83(OT10)	LV11	transparent	X		X	X		X		T
LV116	83(OT10O12)	LV115	opaque	X	X		X		X	Y	O
LV117	83(OT10O3)	LV115	opaque	X	X		X		X	Y	ND
LV118	83(OT10O3T1)	LV117	transparent	X	X		X		X	N	ND
	have 9 O variants of	LV115									
LV119	83-O1	LV1	opaque	X	X		X		X		O
LV120	83-O1T1	LV119	transparent	X	X		X		X	N	T
LV121	83-Onew	LV1	opaque	X	X		X		X		ND
LV122	83-O(n)T1-4	LV121	transparent	X	X		X		X	N	ND

Appendix A: *Mycobacterium intrcellulare* strain Va14 colonial variants isolated.

LV strain numbers	VA14 name	Parent strain	Morphology	Plasmid content of strain 240	68	62	25	18	16	Profile changed	RFLP pattern
LV2	VA14-89(T)	VA14-O	transparent	X		X	X		X	Y	T
LV21	VA14-89(TO)	LV2	opaque	X	X		X		X	Y	O
LV22	89-TO1	LV21	opaque	X	X		X		X		O
LV23	89-TO1T3	LV22	transparent	X	X		X		X	N	T
LV24	89-TO2	LV21	opaque	X	X		X		X		ND
LV25	89-TO2T1	LV24	transparent	X	X		X		X	N	T
LV26	89-TO3	LV2	opaque	X	X		X	X		Y	O
LV27											
LV28	89-TO4	LV21	opaque	X	X		X		X		O
LV29	89-TO4T1	LV28	transparent	X	X		X		X	N	T
LV210	89-TO5	LV21	opaque	X	X		X		X		ND
LV211	89-TO5T1	LV210	transparent	X	X		X		X	N	ND
LV212	89-TO6	LV21	opaque	X	X		X		X		ND
LV213	89-TO6T1	LV212	transparent	X	X		X		X	N	T
	All 9 sibs show same unchanged profile										
LV214	89-T2	LV2	transparent	X		X	X		X		ND
LV215	89-T2O3-5	LV214	opaque	X	X		X		X	Y	O
LV216	89-T3	LV2	transparent	X		X	X		X		ND
LV217	89-T3O1-3	LV216	opaque	X		X	X		X	N	ND
LV218	89-T4	LV2	transparent	X		X	X		X		ND
LV219	89-T4O1-4	LV218	opaque	X		X	X		X	N	ND

Appendix A: *Mycobacterium intrcellulare* strain Va14 colonial variants isolated continued.

Symbols: O, Opaque RFLP pattern; T Transparent RFLP pattern; ND, not done.

Strain Number	E. coli Host	Plasmid number	Vector DNA	Enzymes used	Insert pVT203 size kb	IS1141
VT403	DH5a	pVT300	pUC19	EcoR1	0.6	
VT405	DH5a	pVT301	pUC19	EcoR1	0.6	
VT407	DH5a	pVT302	pUC19	EcoR1	3.7	
VT408	DH5a	pVT303	pUC19	EcoR1	1.4	
VT409	DH5a	pVT304	pUC19	EcoR1	5.5	
VT410	DH5a	pVT305	pUC19	EcoR1	0.7	
VT411	DH5a	pVT306	pUC19	EcoR1	0.62	
VT412	DH5a	pVT307	pUC19	Hind3-EcoR1	2	
to		to	to	to	to	
VT420	DH5a	pVT315	pUC19	Hind3-EcoR1	2	
VT421	DH5a	pVT316	pUC19	EcoR1	3.7	
VT422	DH5a	pVT317	pBR322	Hind3-EcoR1	11	
VT423	DH5a	pVT318	pBR322	Hind3-EcoR1	11	
VT424	DH5a	pVT319	pBR322	Hind3-EcoR1	11,0.5	
VT425	DH5a	pVT320	pBR322	Hind3-EcoR1	12,11,1.4,1.2	
VT426	DH5a	pVT321	pBR322	Hind3-EcoR1	16?, 12	
VT427	DH5a	pVT322	pBR322	Hind3-EcoR1	11,5.2,2.9,2.6,2.0,1.2	
VT428	DH5a	pVT323	pBR322	Hind3-EcoR1	12 = HindIII C	Y
VT429	DH5a	pVT324	pBR322	Hind3-EcoR1	16,11,8.8,2.0,	
VT430	DH5a	pVT325	pBR322	Hind3-EcoR1	2.9,2.7,2.0	
VT431	DH5a	pVT326	pBR322	Hind3-EcoR1	11,4,2.9,2.0	
VT432	DH5a	pVT327	pBR322	Hind3-EcoR1	11,6.2,2.9,2.0	
VT433	DH5a	pVT328	pBR322	Hind3-EcoR1	12	Y
VT434	DH5a	pVT329	pBR322	Hind3-EcoR1	6.6	
VT435	DH5a	pVT330	pBR322	Hind3-EcoR1	2.0, 1.8	
VT436	DH5a	pVT331	pBR322	Hind3-EcoR1	1.8	
VT437	DH5a	pVT332	pBR322	Hind3-EcoR1	1.8, 2.7	

Appendix B: Recombinant plasmids constructed from *M. intracellulare* strain Va14 pVT203.

Strain Number	E. coli Host	Plasmid number	Vector DNA	Enzymes used	Insert pVT203 size kb	Subclone origin	IS1141 ?
VT438	DH5a	pVT333	pUC19	BamHI	5.1	pVT328	Y
VT439	DH5a	pVT334	pBR322	Hind3-EcoR1	6.0, 2.7, 2.0		
VT440	DH5a	pVT335	pBR322	Hind3-EcoR1	10.3, 2.7, 2.0		
VT441	DH5a	pVT336	pBR322	Hind3-EcoR1	2.9, 2.0		
VT442	DH5a	pVT337	pBR322	Hind3-EcoR1	1.8		
VT443	DH5a	pVT338	pBR322	Hind3-EcoR1	5.4		
VT444	DH5a	pVT339	pBR322	Hind3-EcoR1	3.0, 1.8		
VT445	DH5a	pVT340	pBR322	Hind3-EcoR1	12.5, 10.3		
VT446	DH5a	pVT341	pBR322	Hind3-EcoR1	4.2,		
VT447	DH5a	pVT342	pUC19	PstI	3.8 right	pVT328	Y
VT448	DH5a	pVT343	pUC19	PstI	3.8 left	pVT328	Y
VT449	DH5a	pVT344	pUC19	PstI	1.7 right	pVT328	
VT450	DH5a	pVT345	pUC19	PstI	1.7 left	pVT328	
VT451	DH5a	pVT346	pUC19	PstI	1.4 right	pVT328	
VT452	DH5a	pVT347	pUC19	PstI	1.4 left	pVT328	
VT453	XL1-blue	pVT348	pBSK-	PstI-EcoR5	1.2 PstI-PvuII	pVT344	
VT454	XL1-blue	pVT349	pBSK-	PstI-EcoR5	1.4 PstI-PvuII	pVT343	
VT455	XL1-blue	pVT350	pBSK-	PstI-SalI	2.8 Pst-Sal	pVT342	Y
VT456	XL1-blue	pVT351	pVT342	SalI	2.8, 1.0 SalI removed	pVT342	
VT457	XL1-blue	pVT352	pBSK-	Apa	1.9 kb Pst-Apa	pVT350	Y
VT458	XL1-blue	pVT353	pBSK-	Sma	pVT350-sma frag	pVT350	
VT459	XL1-blue	pVT354	pBSK-	Apa Sma	1.6 kb Apa-Sma	pVT350	Y
VT460	XL1-blue	pVT355	pBSK+	ApaSma	1.6 kb Apa-Sma	pVT350	Y
VT461	XL1-blue	pVT356	pBSK-	Pst Bam	0.4 kb PstBam	pVT347	
VT462	XL1-blue	pVT357	pBSK+	PstBam	0.4 kb PstBam	pVT347	
VT463	XL1-blue	pVT358	pBSK-	PstBam	0.7 Kb PstBam	pVT347	
VT464	XL1-blue	pVT359	pVT354	BamBglII	-0.5 kb BamBglII frag	pVT354	Y
VT465	XL1-blue	pVT360	pBSK-	ApaEcoRV	0.5 kb ApaPvuII	pVT333	Y
VT466	XL1-blue	pVT361	pBSK+	ApaEcoRV	0.5 kb ApaPvuII	pVT333	Y
VT467	XL1-blue	pVT362	pBSK+	Bam	0.3 kb BglIIRsaI	pVT354	Y
VT468	XL1-blue	pVT363	pBSK-	BamHIEcoRV	0.4 kb BglIIEcoRV	pVT354	Y
VT469	XL1-blue	pVT364	pBSK+	BamHIEcoRV	0.4 kb BglIIEcoRV	pVT354	Y
VT470	XL1-blue	pVT365	pBSK+	SmaSalI	2.4 kb SmaSal	pVT342	Y
VT471	XL1-blue	pVT366	pBSK-	SmaSalI	2.4 kb SmaSal	pVT342	Y

Appendix B: Recombinant plasmids constructed from pVT203, continued.

Strain Number	E. coli Host	Plasmid number	Vector DNA	Enzymes used	Insert pVT204 size kb	Subclone origin	Hind3C hybridize?
VT500	DH5a	pVT400	pUC19	Hind3-EcoR1	2		
VT501	DH5a	pVT401	pUC19	Hind3-EcoR1	2		
VT502	DH5a	pVT402	pUC19	Hind3-EcoR1	2		
VT503	DH5a	pVT403	pUC19	Hind3-EcoR1	2		
VT504	DH5a	pVT404	pUC19	HindIII	12.5		Y
VT505	DH5a	pVT405	pUC19	HindIII	12.5		Y
VT506	DH5a	pVT406	pUC19	HindIII	12.5, 22		Y
VT507	DH5a	pVT407	pBR322	HindIII	12.5		Y
VT508	DH5a	pVT408	pUC19	Hind3-EcoR1	6.0	pVT405	Y
VT509	DH5a	pVT409	pUC19	Hind3-EcoR1	2.0	pVT405	Y
VT510	DH5a	pVT410	pUC19	Hind3-EcoR1	pVT408-1.7Pst frag	pVT408	Y
VT511	DH5a	pVT411	pUC19	Pst	1.7 kb right	pVT408	Y
VT512	DH5a	pVT412	pUC19	Pst	1.7 kb left	pVT408	Y
VT513	DH5a	pVT413	pUC19	PstEcoRI	1.2 PstPvuII	pVT411	Y

Appendix B: Recombinant plasmids constructed from pVT204.

Strain Number	E. coli Host	Plasmid number	Vector DNA	Enzymes used	Insert size KB	Subclone orgin	IS1141 present?
VT550	DH5a	pVT450	pUC19	EcoRIHindIII	11 kb pVT206		Y
VT551	DH5a	pVT451	pUC19	Bam	6.8 kb Bam pVT205		Y
VT552	DH5a	pVT452	pUC19	Bam	7.6 kbBam pVT205		
VT553	DH5a	pVT453	pUC19	Bam	7.0 kb Bam pVT205		Y
VT554	DH5a	pVT454	pUC19	Bam	7.0 kb Bam pVT205		
VT555	XL1-blue	pVT455	pBSK-	ApaSma	2.2 kb Apa Sma	pVT450	
VT556	XL1-blue	pVT456	pBSK+	ApaSma	2.2 kb Apa Sma	pVT450	
VT557	XL1-blue	pVT457	pBSK-	ApaSma	1.6 kb ApaSma	pVT451	Y
VT558	XL1-blue	pVT458	pBSK+	ApaSma	1.6 kb Apa Sma	pVT451	Y
VT559	XL1-blue	pVT459	pUC19	Sma	pVT450-2.2 kbSma	pVT450	
VT560	DH5a	pVT460	pUC19	EcoRI	6.2 kb of chromosome		Y
VT561	XL1-blue	pVT461	pBSK-	ApaSma	1.5 kb ApaSma	pVT460	
VT562	XL1-blue	pVT462	pBSK+	ApaSma	1.5 kb ApaSma	pVT460	
VT563	XL1-blue	pVT463	pBSK-	ApaSma	1.45 kb ApaSma	pVT460	Y
VT564	XL1-blue	pVT464	pBSK+	ApaSma	1.45 kb ApaSma	pVT460	Y
VT565	XL1-blue	pVT465	pBSK-	ApaSma	1.4 kb ApaSma	pVT460	
VY566	XL1-blue	pVT466	pBSK+	Sma	2.0 Sma	pVT460	Y
VT567	XL1-blue	pVT467	pBSK-	Sma	2.0 Sma	pVT460	Y
VT568	XL1-blue	pVT468	pBSK+	ApaSma	0.76 ApaSma	pVT460	
VT569	XL1-blue	pVT469	pBSK-	ApaSma	0.76 ApaSma	pVT460	
VT570	XL1-blue	pVT470	pBSK+	ApaSma	0.23 ApaSma	pVT460	
VT571	XL1-blue	pVT471	pBSK+	Apa	left 0.37 kb Apa	pVT460	Y
VT572	XL1-blue	pVT472	pBSK+	Apa	right 0.37 kb	pVT460	Y
VT573	XL1-blue	pVT473	pBSK-	Apa	left 0.37 kb	pVT460	Y
VT574	XL1-blue	pVT474	pBSK-	Apa	right 0.37 kb	pVT460	Y
VT580	XL1-blue	pVT480	pBSK-	ApaBam	1.5 kb apaBam	pVT453	Y
VT581	XL1-blue	pVT481	pBSK+	ApaBam	1.5 kb ApaBam	pVT453	Y
VT582	XL1-blue	pVT482	pBSK-	Apa	2.1 kb Apa	pVT450	Y
VT583	XL1-blue	pVT483	pBSK+	Apa	2.1 kb Apa	pVT450	Y
VT584	XL1-blue	pVT484	pBSK-	ApaSal	0.68 kb ApaSal	pVT450	Y
VT585	XL1-blue	pVT485	pBSK+	ApaSal	0.68 kb ApaSal	pVT450	Y
VT586	XL1-blue	pVT486	pBSK+	ApaSal	0.90 kb ApaSal	pVT450	
VT587	XL1-blue	pVT487	pBSK-	ApaSal	0.90 kb ApaSal	pVT450	

Appendix B: Recombinant plasmids constructed from pVT205, pVT206, and the chromosome of *M. intracellulare* strain Va14.

```
Dua5:[Falkinjo]Pvt485.Publish
 -148  AGATCATCTC GACACCCCCT TAGTGTATGA CATATGTAGA CATGAGGCGT
  -98  TGCTTCCATA ACTCGGCTTG TCCTTCCGTT CCCTGCCCTT CCTCGTACTG
  -48  GCCGCTGTGT TTGTGCGTGT GAGGGCGGTG GCTGTCAAAG GCTCGACCCC
    2  CTCTCTGCCA ACCTGGGTTG AGCACCTGCC CCCTGACAAT TAGTGACCGA
   52  GGGCGGGGAT GGAGAGCTTC CCCAAGATGG CGAACACGGT GACCACATTG
  102  GTCCGATCGA CCGGGATCGA AGATCCTGGC CGGATGAACG ACGCTGGGAC
  152  GGAACGTCCC GACCCTGAGG TGCCCGAGCG TGCCCGACGC CGGACGTTCA
  202  CCGCGAAGTA CAAGCTGGAG ATCCTGGCCG CTTACGACGC TGCTCCCGAA
  252  GGCGAGAAGG GTGCGCTGTT GCGCCGGGAG GGGCTGTATT CCACCGACAT
  302  TGTGGCGTGG CGGCGGGCCC   321

Dua5:[Falkinjo]Pvt483.Publish
  319  GGGCCCGCGA CGCCGGCGCG TTGGCTGGCT TGGCCGTTCC GCGCGGACGT
  369  AAGCGGCGCG ACCCACA  385

Dua5:[Falkinjo]Pvt483le.Publish
 1579  TTCAGGGTTG ACAGATTCCG CCTGGTCGAT CGCGCAGCGC GTGGCCTTGA
 1629  CAACCACCAC CCTCACGTTC ACCACAATGT GCGGACGATG GAAGGGACGT
 1679  CTTGGTGCAA TGACGTAATT ACGTCGTCTT GACGGCCCAG GTGTGTAGGC
 1729  GGGGACAGCC GCGCAGATCC ACGTCTGG  1756
```

Appendix C: (1) Sequences from the 16 kb plasmid copy of IS*1141*. Nucleotide numbers correspond to those of IS*1141* reported in Chapter 3. File names are displayed with each partial sequence.

Dua5:[Falkinjo]Pvt458.Publish

```
 319  GGGCCCGCGA CGCCGGCGCG TTGGCTGGCT TGGCCGTTCC GCGCGGACGT
 369  AAGCGGCGCG ACCCACAGGG CGAGCGGATC GCCCGGCTGG AGGCGAAAAA
 419  CACCAACTGG AGCAGGAGCT GGCCAAGACC CGCTTCGTGG TGGACGTCCA
 469  GGCAAAACTG CACGCGCTCT TGGAGACGCT CTCCGAGAGC GCGGACGCCG
 519  AGAACGGGTC GATGAAGTGA GCGACGCAGC GATCAGCGAG CTGGCGCCCA
 569  AGATC  573
```

Appendix C: (2) Sequences from 18 kb plasmid copy of IS*1141*. Nucleotide numbers correspond to those of IS*1141* reported in Chapter 3. File names are displayed with each partial sequence.

```
Dua5:[Falkinjo]Pvt472.Publish
-69  CCACTTTCCT CGCCGGTCAA GGACTTTTGA CGCGGTTGTG CCTTTCTCGT
-19  CGCTGATCGG TCAGCGCGAC CCTCTCTGCC AACCTGGGTT GAGGCACCTG
 31  CCCCCTGACA ATTAGTGACC CGAGGGCGGG GATGGAGAGC TTCCCCAAGG
 81  ATGGCGAACA C  91

 Dua5:[Falkinjo]Pvt464.Publish
319  GGGCCCGCGA CGCCGGCGCG TTGGCTGGCT TGGCCGTTCC GCGCGGACGT
369  AAGCGGCGCG ACCCACAGGG CGAGCGGATC GCCCGGCTGG AGGCCGAAAA
419  ACACCAACTG GAGCAGGAGC TGGCCAAGAC CCGCTTCGTG GTGGACGTCC
469  AGGCAAAACT   478

Dua5:[Falkinjo]Pvt463.Publish
1508  AGCGGCTCAT GGATCAACAA ACCAGACGAC ACCGAGGAGG CCATTCAGTA
1558  AATACCCTCA ACAGTGCCTC ATTCAGGTTG ACAGATTCGC GACGCTGTGG
1608  TGTCATGCCA CAACGGGCGG TGAGCCCGTC ACACGTCACG ACAGCGCGCT
1658  ACGTTGCGGC GCCGAAACAG AGGCGACGAG CCCATGACAG AGCACAGCAT
1708  GTGCTGCCTA TGCGCCGCAC GCGTTGCTTT GGCCCGGG  1745
```

Appendix C: (3) Sequences from 6 kb *Eco*RI copy of IS*1141*. *Nucleotide numbers correspond to those of IS1141* reported in Chapter 3. File names are displayed with each partial sequence.

CURRICULUM VITAE

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Education

1988 - 1993 Ph.D. in Biology (Microbiology) Virginia Polytechnic Institute & State University, Blacksburg, VA. Dissertation title: Insertion sequence IS*1141:* discovery, charactactization, and association with *Mycobaterium intracellulare* colonial variation.

1986 - 1987 Graduate Courses, Hollins College, Hollins VA.

1980 - 1984 B.S. in Secondary Science Education, Univ. of Virginia, Charlottesville, VA.

Professional Experience

1989 - Present Graduate Research Assistant: Biology Department, VPI & SU, Blacksburg VA. Laboratory of Joseph O. Falkinham, III, Ph.D.

1988 - 1991 Graduate Teaching Assistant: Biology Department, VPI & SU, Blacksburg VA. Principles of Biology Laboratory and Molecular Genetics Laboratory.

1987 Volunteer Research Assistant: Plant Physiology Pathology and Weed Science, VPI & SU, Blacksburg VA. Laboratory of George H. Lacy, Ph.D.

1986 - 1987 Curriculum Development Staff: Roanoke City Public Schools, Roanoke, VA.

1984 - 1988 Biology and Advanced Placement Biology Teacher: Roanoke City Public Schools, Roanoke VA.

Honors and Awards

1992 - 1993	State Tuition Scholarship
1992	Graduate Student Service Award
1991 - 1992	State Tuition Scholarship
1990 - 1991	State Tuition Scholarship
1988 Fall	State Tuition Scholarship
1987	Outstanding Teacher Award, The University of Chicago, Chicago, IL.

Grants and Fellowships

1992 Grant "Use of a transposable element as a epidemiologic marker for *Mycobacterium intracellulare.*" Sigma-Xi Grant-in-Aid of Research. April 1992 - March 1993 ($400 + matching amount from Dept.)

1992 Graduate Student Assembly Travel Fund Grant, ($250)

1991 Commonwealth Fellowship from the State Council of Higher Education for Virginia, ($5000).

Publications and Reports

A. Papers in Refereed Journals

C. Neal Stewart, Jr. and Laura E. Via. 1993. A simple CTAB DNA isolation technique useful for RAPD fingerprinting and other DNA amplifications. BioTechniques. *in press.*

Laura E. Via and Joseph O. Falkinham, III. 1991. A simple method for isolating restriction fragments of large plasmids recovered from bacteria with multiple plasmids. BioTechniques 11:442-444.

B. Manuscripts in Preparation

Laura E. Via, C. Neal Stewart, Jr. and Joseph O. Falkinham, III. Typing of *Mycobacterium avium* complex strains by random amplified polymorphic DNA (RADP) fingerprinting. To be submitted to Tubercle and Lung Disease.

Laura E. Via and Joseph O. Falkinham, III. Comparison of methods for isolation of *Mycobacterium avium* complex DNA for the polymerase chain reaction. To be submitted to J. Microbiol. Methods.

Laura E. Via and Joseph O. Falkinham, III. Discovery and characterization of IS1141 in *Mycobacterium intracellulare* strain Va14. To be submitted to Molecular Microbiol.

Laura E. Via and Joseph O. Falkinham, III. Association between colonial variation in *Mycobacterium intracellulare* and changes in IS1141 restriction fragment length polymorphism patterns. To be submitted to Molecular Microbiol.

Laura E. Via, and Joseph O. Falkinham, III. Distribution of IS1141 in *Mycobacterium avium* complex. To be submitted to Molecular Microbiol.

C. Abstracts Published

Laura E. Via, C. Neal Stewart, Jr., and Joseph O. Falkinham, III. Utility of random amplified polymorphic DNA analysis in *Mycobacterium avium* complex epidemiology. Frontiers In Mycobacteriology: *M. avium*, the Modern Epidemic. Symposium on Oct. 15-19, 1992 in Vail, Colorado.

Laura E. Via and Joseph O. Falkinham, III. 1992. Colonial variation in *Mycobacterium intracellulare* correlates with transposition. Plasmid **28**:179.

Laura E. Via and Joseph O. Falkinham, III. 1992. Transposition and colonial variation in *Mycobacterium intracellulare*. U-45, p.173. Abstr. 92nd Annu. Meet. Am. Soc. Microbiol. 1992.

Laura E. Via and Joseph O. Falkinham, III. 1991. Colonial variation and changes in plasmid DNA profiles in *Mycobacterium avium* complex. Plasmid **25**:159-160.

D. Abstracts Submitted

Laura E. Via and Joseph O. Falkinham, III. Discovery and distribution of insertion sequence IS1141 in *Mycobacterium intracellulare*. Submitted for the 93rd Annu. Meet. Am. Soc. Microbiol. 1993. May 16 to 20th in Atlanta, Georgia.

Invited Seminars

VPI and SU Molecular and Cellular Biology Seminar, Nov. 6, 1992. "Discovery and characterization of IS1141 from *Mycobacterium intracellulare* and IS1141's relationship to colonial variation."

VPI and SU Training the Future Professorate Seminar, Aug. 17-19, 1992. "Dual role of the Graduate Student."

Community and University Service

1992 - present	Member of Montgomery County Public Schools "FOCUS 2006" Strategic Planning Commission Academic Preparation Task Force.
1992 - present	Member of University Commission of Human Rights and Social Responsibility.

1992 - 1993	Chief Investigator for Graduate Honor System. Responsibilities include supervision and training of 5 investigators, case review and assisting Chief Justice.
1990 - 1992	Investigator for Graduate Honor System (1 of 2 appointed). Service included delivering orientation lectures, convening investigative boards and presenting evidence to judicial panels.
1990 - 1992	Biology Department Graduate Advisory Committee Member, Chairman 1991 - 1992.
1990 - 1991	Graduate Honor System Constitution Committee
1990 - 1991	VPI Biology Graduate Student Mentor Program

Professional Memberships

American Society of Microbiology
National Association of Biology Teachers
Phi Kappa Phi, VPI & SU Chapter
Phi Sigma Society, Alpha Phi Chapter