

RELATIONSHIP OF PLASMIDS IN
MYCOBACTERIUM AVIUM, MYCOBACTERIUM INTRACELLULARE,
AND MYCOBACTERIUM SCROFULACEUM

by

Markus Thomas Jucker

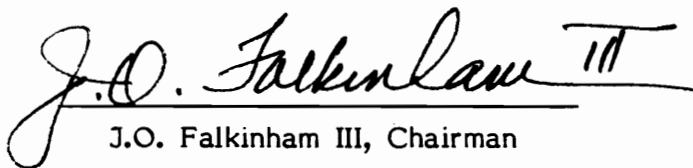
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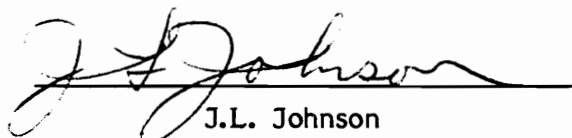
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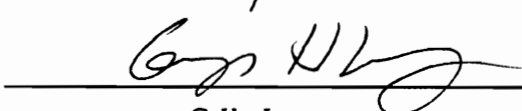
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Biology

(ABSTRACT)

Bacteria of the Mycobacterium avium, M. intracellulare, and M. scrofulaceum group (MAIS) are opportunistic human pathogens and widespread in the environment. The first objective of this study was to demonstrate that plasmids from clinical isolates are closely related to plasmids from environmental MAIS isolates. A 12.9 kb plasmid, pVT2, from a clinical M. avium isolate, MD1, was cloned and used as a DNA probe to examine the relationship of MAIS plasmids. The pVT2 probe hybridized with plasmids isolated from MAIS strains from the environment, from patients without AIDS with pulmonary infections, and from AIDS patients with disseminated MAIS infections. Similar results were seen with a second probe derived from pLR7, a 15.3 kb plasmid from clinical M. intracellulare strain LR113. The similarity of plasmids from environmental and clinical isolates supports the hypothesis that the environment is a source of MAIS infection in humans.

Also, plasmid DNA probes were developed and used to show that MAIS plasmids could be placed into four distinct groups based on hybridization. Plasmids in Group 1 were the smallest and most common (22 of 39 plasmid-bearing isolates). Plasmids in Group 2 were also common (16 of 39 plasmid-

bearing isolates), and highly correlated with AIDS isolates. Plasmids in Groups 3 and 4 occurred much less frequently. The four plasmid homology groups represent potentially useful epidemiological markers.

The second objective of this study was to determine the nature of conserved DNA shared among related plasmids of different sizes in homology Group 1. Restriction analysis, hybridization, thermal stability, and DNA sequencing experiments showed that the Group 1 plasmids shared two regions of conserved DNA, interspersed with nonshared DNA. The conserved regions may provide essential plasmid replication functions for the construction of a MAIS cloning vector. Group 1 plasmids appeared to be products of several insertion and/or deletion events, and provide a model of active plasmid evolution. These results filled a gap in our basic knowledge of MAIS plasmid biology and are of significance to our understanding of MAIS epidemiology, and thus have implications on public health measures.

Dedicated to

Renée Jucker

with love and gratitude

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CHAPTER 1

INTRODUCTION

Bacteria of the Mycobacterium avium, M. intracellulare, and M. scrofulaceum group (MAIS) are slow-growing, acid-fast-staining rods with a mole-percent guanosine + cytosine of 68%. MAIS have a hydrophobic cell envelope rich in long-chain waxes that evidently forms a permeability barrier which provides a high level of non-specific resistance to antibiotics, (Mizuguchi, et al., 1983), heavy metals (Falkinham, et al., 1984), and chlorination (duMoulin and Stottmeier, 1986).

MAIS are opportunistic human pathogens, and have gained increasing attention as agents of pulmonary infections with symptoms clinically indistinguishable from tuberculosis (Wolinsky, 1979) and disseminated infections in AIDS patients (Blaser and Cohn, 1986). Approximately 25% of AIDS patients have disseminated MAIS infection. (Blaser and Cohn, 1986). MAIS cause disease in immunosuppressed patients, patients with previous lung lesions, alcoholics, the elderly, and children, as well as AIDS patients (Wolinsky, 1979). Because the number of persons who are susceptible to MAIS infection is increasing, the number of MAIS infections is also expected to increase (duMoulin and Stottmeier, 1986; Ellner, 1991). MAIS also infect numerous domesticated animals, including rhesus monkeys (duMoulin and Stottmeier, 1986; Masaki, et al., 1989), and are widespread contaminants in plant tissue cultures (Taber, et al., 1991).

MAIS are virtually ubiquitous in the environment and person-to-person transmission has never been demonstrated (Wolinsky, 1979). Epidemiological studies have shown that the environment is a source of human infection (Falkinham, et al., 1980) via aerosolization from natural waters (Wendt, et al., 1980; Fry, et al., 1986; Meissner and Falkinham, 1986) and water distribution systems (duMoulin, et al., 1988; Burns, et al., 1991). The

distribution of MAIS in the southeastern United States correlates with the high frequency of persons showing evidence of MAIS infection (Edwards, et al., 1969) and MAIS-infected persons living in these areas (Good and Snider, 1980).

Plasmids are common in MAIS, and most plasmid-bearing strains have two to six plasmids ranging in size from 10.5 to 345 kb in size (Meissner and Falkinham, 1986) and comprising up to 9% of the total DNA. Nearly all MAIS isolated from AIDS patients have plasmids (Crawford and Bates, 1986) and plasmids also occur frequently in isolates from patients without AIDS and in aerosol isolates (Meissner and Falkinham, 1986). Thus, while no mechanism of plasmid-associated virulence has been documented, there is a strong epidemiological suggestion that warrants study.

Meissner and Falkinham (1986) showed that many plasmids from environmental MAIS isolates appear to be identical to clinical isolates when sized in agarose gels of uncut plasmid DNA. This observation supports the hypothesis that the environment is a source of human infection. However, further evidence was needed to prove that these environmental and clinical plasmids are related. Such evidence would have a potential impact on public health measures.

Serotyping, phage typing, biotyping, and restriction fragment length polymorphism (RFLP) have been applied with some success as epidemiological markers in MAIS. However, all these techniques suffer practical limitations: 1) A significant number of MAIS isolates cannot be serotyped or autoagglutinate (Brennan, et al., 1982). Also, AIDS isolates are limited to a narrow spectrum of serotypes (Yakrus and Good, 1990). 2) Many clinical (66%) and environmental (97%) MAIS isolates are phage resistant and cannot be phage

typed (Crawford and Bates, 1985). 3) Biotyping is poorly developed and has not been applied to clinical isolates. 4) RFLP typing offers promise but presently only weak discrimination is possible due to the predominance of a single class (Boddinghaus, et al., 1990; Chiodini, 1990). Therefore, one of the goals of this study was to establish the utility of plasmid typing in MAIS as an epidemiological tool.

MAIS plasmids have been shown to encode restriction-modification (Crawford et al., 1981), mercury resistance (Meissner and Falkinham, 1984), cadmium resistance (Erardi, et al., 1987), and in M. chelonae, morpholine degradation (Waterhouse, et al., 1991). It is likely that other potentially useful degradative abilities will prove to be plasmid-encoded, as is the case in other genera, such as Pseudomonas. It has been suggested that MAIS plasmids are associated with virulence characteristics as in other genera (Crawford and Bates, 1986). Meissner and Falkinham (1986) found that MAIS isolated from aerosols were more likely to contain plasmids (70% of aerosol isolates had plasmids) and were more hydrophobic than other environmental MAIS isolates. Hydrophobicity can be expected to be a virulence characteristic in MAIS as it is in other bacteria, and thus plasmid-bearing aerosol isolates may contain plasmid-encoded virulence characteristics. It has also been suggested that MAIS strains bearing plasmids are able to inhibit the oxidative burst of macrophages in which they reside (Gangadharam, et al., 1988).

Using a DNA probe prepared from plasmid pLR7 from a clinical M. avium isolate, Crawford and Bates (1986) found that strains isolated from AIDS patients harbored plasmids of different sizes that hybridized with pLR7. However, many additional plasmids did not hybridize with pLR7. This

result led to the formulation of two hypotheses: 1) MAIS plasmids can be placed into groups on the basis of hybridization with MAIS plasmid DNA probes. 2) The plasmids within a group, although differing in size, share conserved DNA regions. The related plasmids could share DNA as: (1) a discrete conserved region(s), (2) a repetitive sequence(s) inserted into otherwise unrelated plasmids, or (3) long, but relatively poorly conserved regions.

It seems likely that DNA regions that are conserved in plasmids of different sizes would encode biologically significant functions such as genes for DNA replication and segregation, plasmid maintenance, plasmid incompatibility, hydrophobicity, or virulence. In various invasive Salmonella serovars, for example, the vir (virulence), repB (maintenance), and par (maintenance) regions are highly conserved in a group of plasmids of 50 to 100 kb (Tinge and Curtiss, 1990). One of the objectives of this work was to analyze conserved regions in MAIS plasmids to provide the basis for the development of MAIS cloning vectors. Currently no practical system for MAIS genetic analysis is available. A cloning vector, coupled with a technique for transforming MAIS, or a practical MAIS phagemid, would be a powerful tool for MAIS research. Cloning vectors have been developed for use in M. smegmatis, a rapid-growing mycobacterium, exploiting replication regions from a mycobacteriophage (Jacobs, et al., 1987), and for M. bovis strain BCG, using a region from a M. scrofulaceum plasmid that encodes autonomous replication (Goto, et al., (1991). This approach should succeed in MAIS as well, once the requisite essential plasmid regions are discovered and the hurdle of MAIS transformation is overcome.

Objectives

The main objectives of this study are summarized below.

- 1) Examine whether plasmids from clinical and environmental MAIS isolates are closely related by the use of plasmid DNA probes.
- 2) Analyze the nature of the conserved regions in plasmids which are related by hybridization but are of unequal sizes.

CHAPTER 2

EVIDENCE FOR TWO DNA HOMOLOGY GROUPS AMONG PLASMIDS IN MYCOBACTERIUM AVIUM, M. INTRACELLULARE, AND M. SCROFULACEUM

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INTRODUCTION

Members of the Mycobacterium avium, M. intracellulare, and M. scrofulaceum group (MAIS) are opportunistic human pathogens (Wolinsky, 1979). The recent increase in MAIS infections is due, in part, to the fact that a substantial proportion of patients with acquired immunodeficiency syndrome (AIDS) have disseminated MAIS infections (Blaser and Cohn, 1986). Studies have indicated that the source of human MAIS infection is the environment (Falkinham, et al., 1980), including hospital hot water systems (duMoulin, et al., 1988) via aerosolization (Wendt, et al., 1980; Parker, et al., 1983), based on the identity of clinical and aerosol isolates (Fry, et al., 1986; Meissner and Falkinham, 1986). Although the functions of most MAIS plasmids remain cryptic, plasmids have been shown to encode functions involved in restriction and modification (Crawford, et al., 1981), mercury (Meissner and Falkinham, 1984) and copper resistance (Erardi, et al., 1987) and virulence (Gangadharam, et al., 1988). Though plasmids of the same molecular weight have been found in both clinical and environmental MAIS strains (Meissner and Falkinham, 1986), it has not been proven that such plasmids are identical (i.e. by DNA-DNA hybridization).

Using a DNA probe prepared from a plasmid from a clinical M. avium isolate (pLR7) (Crawford and Bates, 1984), Crawford and Bates (1986) found that MAIS strains isolated from persons with AIDS harbored plasmids related to pLR7. However, environmental isolates were not examined and not all small plasmids (i.e. less than 26 kb) from these strains hybridized with their probe. In strains which contained two small plasmids, pLR7 hybridized only with the larger of the two (Crawford and Bates, 1986).

To examine the genetic relatedness of environmental and human MAIS strains, and because of the possible role of plasmid-encoded genes in virulence, we sought to determine whether homology exists between plasmids of environmental and clinical strains. In this report we describe the use of a cloned M. avium plasmid, pVT2, as well as pLR7 (Crawford and Bates, 1986), as DNA probes to demonstrate that small MAIS plasmids from clinical and environmental strains were related and that MAIS plasmids could be divided into at least three distinct groups based on DNA-DNA homology.

METHODS

Bacteria. A total of thirty-nine plasmid-bearing M. avium, M. intracellulare, and M. scrofulaceum (MAIS) strains were used in this study (Table 1). The clinical and environmental isolates had been isolated in the southeastern United States and included those employed in earlier studies (Meissner and Falkinham, 1986). MAIS strains 535, 541, 542, and 543, isolated from persons with AIDS and originally obtained from the Centers for Disease Control, Atlanta, Georgia were kindly provided by Jack Crawford of the John L. McClellan Memorial Veterans Hospital, Little Rock, Arkansas. Dr. Crawford also provided M. avium strains LR25 and LR113 and Escherichia coli strain HB101 containing pJC20, which is a pBR322 derivative containing the entire sequence of pLR7, the single 15.3 kb plasmid in LR113 (Crawford and Bates, 1984). Strains 2812 and 2816 were isolated from AIDS patients from the Westchester, New York jail and W1158 and W1233 from soil collected in Westchester County. These strains were isolated and identified by our late colleague, Dr. Howard Gruft of the New York State Department of Health.

Plasmid DNA isolation. Mycobacteria were grown to late log phase in Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, MD) containing 10% (vol/vol) oleic acid - albumin enrichment and treated with D-cycloserine and ampicillin before lysis as described by Crawford and Bates (Crawford and Bates, 1979). Crude plasmid DNA was isolated by a modification of the Kado and Liu (1981) alkaline lysis procedure as described earlier (Meissner and Falkinham, 1986). Mycobacterial plasmid DNA was purified for restriction digestion as follows: Cells from 100 ml of culture were grown and

Table 1. DNA homologies and apparent molecular weights of undigested plasmids recovered from clinical and environmental MAIS isolates.

Strain	Source	Plasmids (kb)	Reference
Human			
GA1	Non-AIDS	13.5 ¹ 22 42 33 155	Meissner and Falkinham, 1986
GA2	Non-AIDS	13.5 ¹	"
GA9	Non-AIDS	13.5 16.6 44 155	"
LR25	Non-AIDS	17.0 ² 28 [†] 162	Crawford et al., 1981
LR113	Non-AIDS	15.3 ²	Crawford and Bates, 1984
MD1	Non-AIDS	12.9 ¹	Meissner and Falkinham, 1986
MD2	Non-AIDS	20.5 31	"
MD3	Non-AIDS	13.5 ¹ 19.7 ²	"
MD12	Non-AIDS	19.1 ² 25	"
MD17	Non-AIDS	17.5 18.5 28 [†]	"
MD22	Non-AIDS	13.5 ¹ 16.9 22.3 ² 160	"
SC2	Non-AIDS	16.3 31	"
SC7	Non-AIDS	13.5 ¹ 17.6 25 29 160	"
VA2	Non-AIDS	13.5 ¹ 17.4 25 39 54 135	"
VA3	Non-AIDS	13.5 ¹ 17.0 ² 155	"
VA10	Non-AIDS	13.5 ¹ 16.4 24 62 155 230	"
VA14	Non-AIDS	15.6 28 58 245	"
535	AIDS	13.5 ¹ 17.6 ²	Crawford and Bates, 1986
541	AIDS	13.5 ¹ 24.5 ² 160	"
542	AIDS	13.5 ¹ 19.5 ²	"
549	AIDS	15.6 ²	"
2812	AIDS	15.3 ¹ 17.6 ²	this study
2816	AIDS	15.3 ¹ 19.3 ²	this study

¹ Hybridized with pVT2.

² Hybridized with pLR7.

[†] Showed weak hybridization with pVT2.

Table 1, continued.

Strain	Source	Plasmids (kb)	Reference
Environmental			
CL25	chicken litter	13.5 ¹ 160	Falkinham et al., 1989
CL26	chicken litter	13.5 ¹ 17.7 22 24 26 170	"
CL28	chicken litter	13.5 ¹ 15.5 160	"
DE20	water	13.5 ¹ 17.6 ² 160 180	Falkinham et al., 1980
5S	aerosol	13.5 ¹ 19.9	Wendt et al., 1980
11S	aerosol	19.6 ² 155	"
12S	aerosol	17.0 ² 24	"
SR353	water	22.5	Falkinham et al., 1980
SR481	water	162	"
SR493	water	162	"
SR522	water	160	"
W257	water	38	"
W258	water	21	"
W262	water	13.3 ¹ 150 170 300	Meissner and Falkinham, 1984
W1158	soil	15.3 ¹ 21 ²	this study
W1233	soil	15.3 ¹	this study

¹ Hybridized with pVT2.

² Hybridized with pLR7.

[†] Showed weak hybridization with pVT2.

treated with antibiotics as described above, harvested, and resuspended in 2.0 ml of TE buffer (Maniatis, et al., 1982) in 50 ml polyethylene centrifuge tubes. Six ml of lysing solution (3% sodium dodecyl sulfate, 50 mM Tris, pH 12.3; Kado and Liu, 1981) was added and mixed by rotation of the tubes at 4 rpm for 5 min. Next, tubes were incubated in a 60°C water bath for 20 minutes and allowed to cool at room temperature for 15 minutes. After extraction with phenol-salt (Meissner and Falkinham, 1986) the aqueous phase was extracted with phenol-chloroform and then with chloroform-isoamyl alcohol (Maniatis, et al., 1982). After centrifugation, one half volume of 7.5M ammonium acetate was added to the aqueous supernatant. The mixture was incubated on ice for 20 minutes and centrifuged at 15,000 x g maximum for 25 minutes. The pellet, which contains contaminants that can inhibit DNA-modifying enzymes (J.L. Johnson, personal communication) was discarded and the plasmid DNA was precipitated from the supernatant with ethanol (Maniatis, et al., 1982). The pellet was resuspended in 4 ml of TE and the precipitations with ammonium acetate and with ethanol were repeated twice as described above. To isolate the smallest plasmid from strain VA3 for restriction analysis, plasmid DNA was purified as described above. The smallest plasmid was then separated by electrophoresis in low melting point agarose (Fisher Scientific; Fairlawn NJ) (Maniatis, et al., 1982) and isolated and purified with a Geneclean kit (Bio 101; La Jolla CA) according to the manufacturer's instructions. Plasmids were separated by electrophoresis in 0.7% horizontal agarose gels in Tris-acetate buffer (Maniatis, et al., 1982), using plasmids from strain LR25 (Crawford and Bates, 1979) and LR113 (Crawford and Bates, 1984) as covalently closed circular plasmid DNA molecular weight markers.

Plasmid DNA hybridization. Ethidium bromide-stained gels (Maniatis, et al., 1982) were exposed to UV (302 nm, Photodyne, New Berlin, Wis.) for six minutes to induce nicking, and then DNA was transferred to Zeta-Probe membrane by alkaline blotting according to the manufacturer's instructions (Bio-Rad Laboratories, Inc.; Richmond CA). Hybridization of DNA probes with MAIS plasmids on Zeta-Probe membranes was performed at 50°C in 50% (vol/vol) formamide according to Bio-Rad's instructions. Some Southern transfers were sequentially hybridized with both plasmid probes. Those Southern transfers were air-dried at room temperature before autoradiography. The first probe was removed from the filter by two exposures to boiling 0.1X SSC, 0.5% SDS for 30 min (Maniatis, et al., 1982). The transfer was autoradiographed before hybridization with the second probe to confirm that removal of the first hybridization probe had occurred. Autoradiographs were exposed at room temperature on Kodak X-Omat film.

Construction of plasmid DNA probes. Plasmid pVT2 (12.9 kb) was isolated from M. avium strain MD1 and mapped by restriction analysis (Figure 1). The plasmid was cleaved with EcoRI and HindIII and the two resulting fragments (4.2 kb and 8.7 kb) were inserted into pUC19 (Yannisch-Perron, et al., 1985) by standard techniques (Maniatis, et al., 1982) to produce the recombinant plasmids pVT100 and pVT101, respectively. The two pVT2 fragments and the pLR7 insert in pJC20 were removed from their respective recombinant plasmids by endonuclease restriction digestion and purified by low melting point agarose electrophoresis followed by phenol extraction (Maniatis, et al., 1982), and radiolabeled with ³⁵S-dCTP (New England Nuclear; Cambridge MA) using a random primer DNA labeling kit (Boehringer-Mannheim, Inc.; Indianapolis IN)

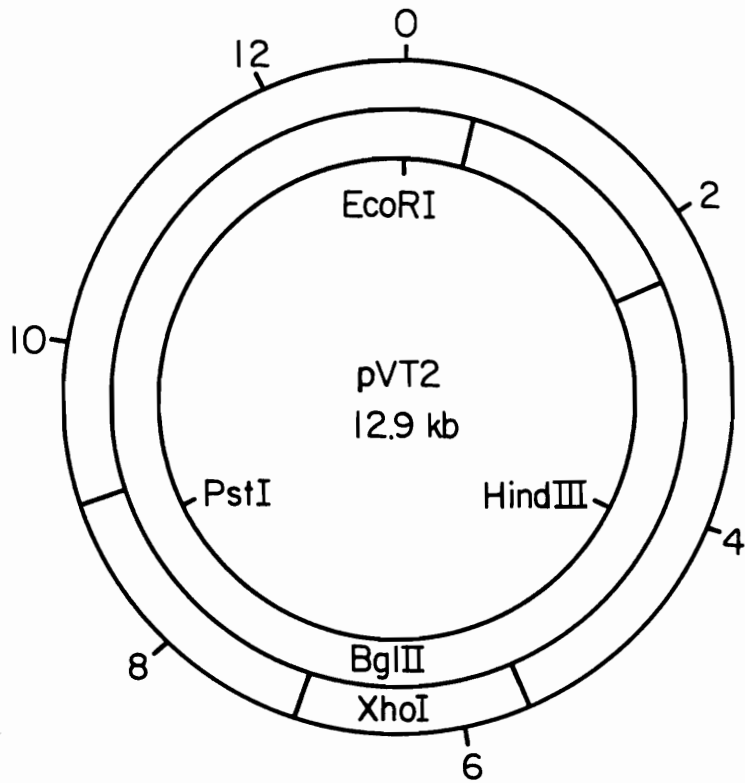


Figure 1. Restriction map of *M. avium* plasmid pVT2. There are no sites for BamHI and XbaI. Coordinates are in kilobases. EcoRI was chosen arbitrarily as zero.

according to the manufacturer's instructions. The two pVT2 fragments were pooled and radiolabeled in one reaction.

RESULTS

Plasmids ranging in size from 12.9 to 300 kb (Figure 2A) from 23 clinical and 16 environmental plasmid-bearing MAIS strains were hybridized with the M. avium plasmids pVT2 and pLR7 (Figure 2). The probe prepared from pVT2 hybridized with 13.5 kb and 15.3 kb plasmids (Figure 2B) found in 15 clinical (65%) and in seven environmental (44%) isolates (Tables 1 and 2). The two smallest bands in lane 3 of the ethidium bromide-stained gel (Figure 2A) are faint. However, they are clearly visible in the Southern blot (Figure 2B, lane 3). No plasmids larger than 15.3 kb hybridized with pVT2, though the 28 kb plasmids in strains LR25 (Figure 2B) and MD17 (data not shown) hybridized weakly. The probe prepared from pLR7 hybridized with plasmids of at least six distinct sizes ranging from 15.3 to 24.7 kb (Figure 2C) recovered from 12 clinical (52%) and four environmental (25%) strains (Tables 1 and 2). No plasmids larger than 25 kb hybridized with the pLR7 probe. Eighteen strains contained plasmids within the 15.3 - 25 kb range which did not hybridize with either probe (Figure 2B and C). Plasmids pVT2 and pLR7 did not hybridize with each other. Three clinical (non-AIDS) (18%), five AIDS (83%), and two environmental (12%) strains contained both pVT2- and pLR7-related plasmids (Table 1, Figure 2B and C). Of the six AIDS isolates, five (83%) carried a plasmid which hybridized with pVT2 and all carried plasmids which hybridized with pLR7 (Table 2), in agreement with Crawford and Bates (1986).

The plasmid preparation from DE20 yielded two bands which hybridized with the pVT2 probe (Figure 3A and B). To demonstrate that the two bands were topoisomeric forms of a single plasmid, this preparation was digested with EcoRI, subjected to electrophoresis and Southern transfer, and

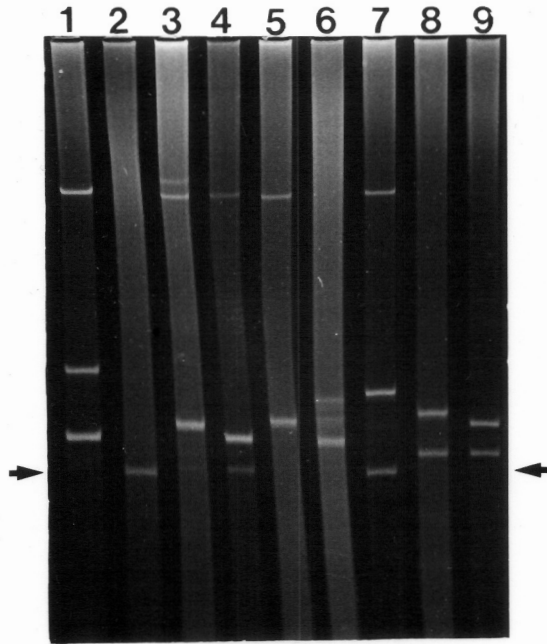


Figure 2A. Agarose gel electrophoresis of crude plasmid DNA prepared from human and environmental MAIS isolates. Lane 1, strain LR25, which carries plasmids of 162, 28, and 17 kb, from top to bottom; Lane 2, strain MD1, carrying pVT2; Lane 3, DE20; Lane 4, VA3; Lane 5, 11S; Lane 6, 12S; Lane 7, 541; Lane 8, W1158; Lane 9, 2816. The upper and lower arrows indicate 17.0 and 13.5 kb plasmids (respectively) and show the orientation of the autoradiographs relative to the gel. The two smallest bands in lane 3 are faint.

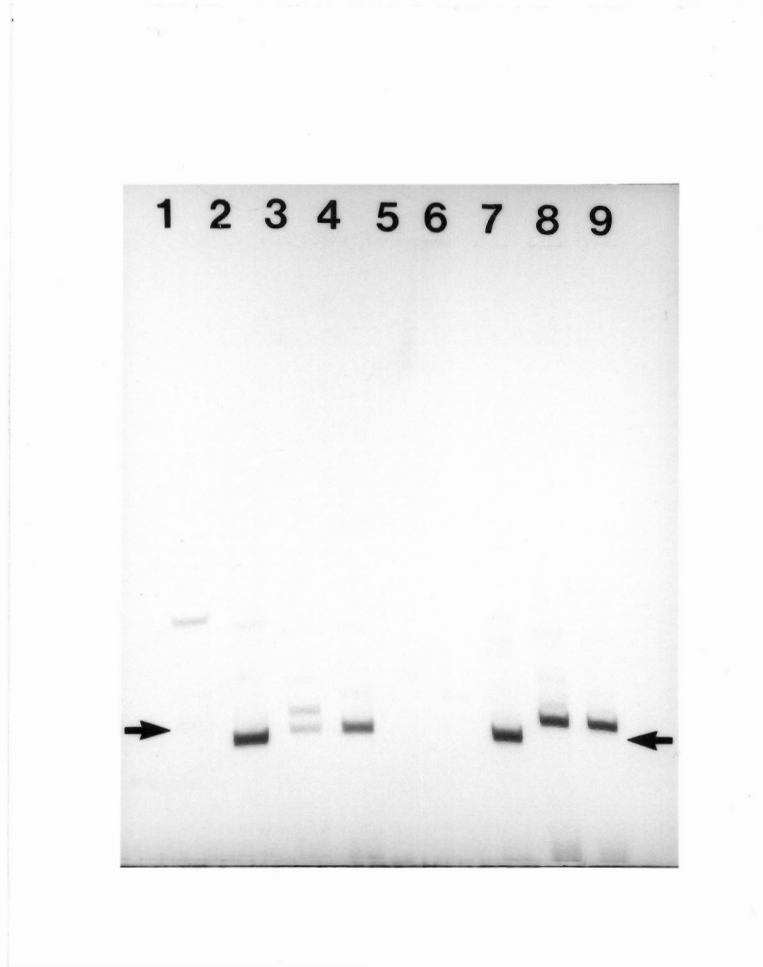


Figure 2B. Autoradiograph of a Southern transfer of gel 2A, which was hybridized with a mixture of the radiolabeled pVT2-derived inserts of recombinant plasmids pVT100 and pVT101 (pVT2::pUC19 chimeras), which together carry the complete pVT2 sequence.

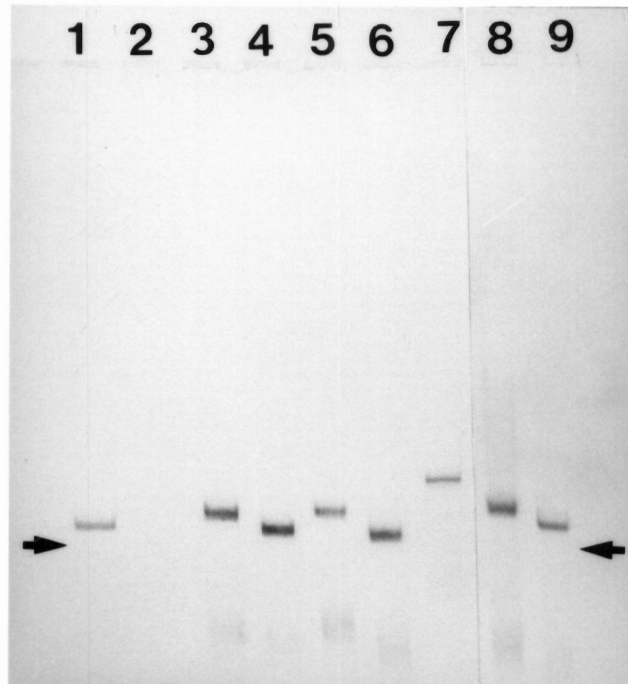


Figure 2C. Autoradiograph of a Southern transfer of a gel identical to 2A, which was hybridized with the radiolabeled complete pLR7 insert of recombinant plasmid pJC20.

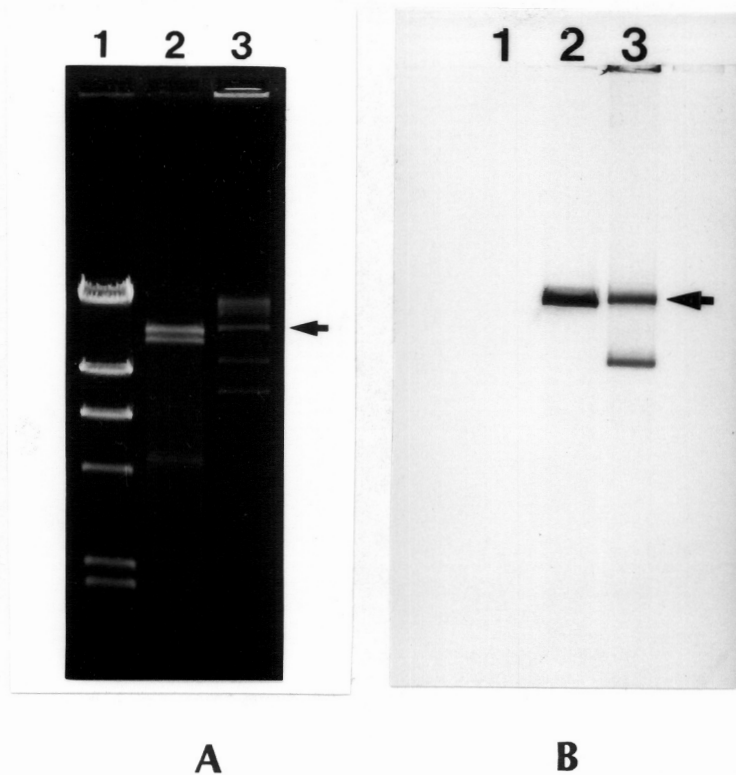


Figure 3. (A) Agarose gel electrophoresis of purified plasmids isolated from strain DE20. Lane 1, HindIII digest of lambda DNA, with molecular weights of 23.2, 9.6, 4.6, 2.3, 2.0, and 0.56 kb., from top to bottom; Lane 2, EcoRI digest of plasmids from strain DE20; Lane 3, uncut plasmid preparation. (B) Autoradiograph of a Southern transfer of gel A, which was hybridized with the radiolabeled pVT2-derived inserts of plasmids pVT100 and pVT101. The arrow indicates orientation of the autoradiograph relative to the gel.

hybridized with the pVT2 probe. A single pVT2-related band resulted (Figure 3A and B). Multiple bands which hybridized with the pVT2 probe have also been observed in gels of uncut plasmids from strains MD1, MD22, and VA3. These preparations also yielded single pVT2-hybridizing bands upon cleavage with EcoRI (data not shown).

To assess how large a region of DNA sequence identity was shared by the pVT2-related plasmids, SalI restriction fragments of the 12.9 kb (pVT2), 13.5 kb, and 15.3 kb plasmids from strains MD1, VA3, and W1233 (respectively) were separated by electrophoresis (Figure 4A) and hybridized with the radiolabeled pVT2 probe (Figure 4B). All SalI digests shared 1.0 kb fragments. The digests of pVT2 and the 13.5 kb plasmid shared 2.2 kb fragments. The digests of the 13.5 and 15.3 kb plasmids shared fragments of 4.8, 3.5, and 0.85 kb. Probe pVT2 hybridized with all SalI restriction fragments from the three plasmids except the 0.85 kb fragment from the 13.5 and 15.3 kb plasmids. That smallest fragment is faint in the ethidium bromide-stained gel (Figure 4A, lanes 3 and 4). The small amount of DNA may have limited hybridization (Figure 4B).

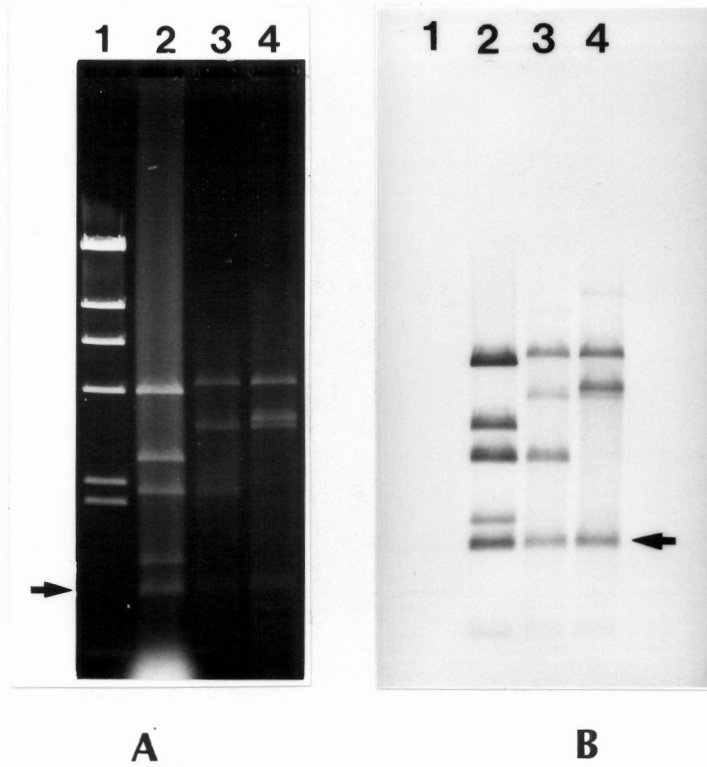


Figure 4. (A) Agarose gel electrophoresis of *Sal*I digests of pVT2-related plasmids. Lane 1, *Hind*III digest of lambda DNA; Lane 2, plasmid pVT2 from strain MD1; Lane 3, the 13.5 kb plasmid from strain VA3; Lane 4, the 15.3 kb plasmid from strain W1233. The smallest band in lanes 3 and 4, below the arrow, is faint. (B) Autoradiograph of a Southern transfer of gel A, which was hybridized with the radiolabeled pVT2-derived inserts of plasmids pVT100 and pVT101. The arrow indicates the orientation of the autoradiograph relative to the gel.

DISCUSSION

Hybridization between plasmids from 23 clinical and 16 environmental plasmid-bearing M. avium, M. intracellulare, and M. scrofulaceum (MAIS) strains and the small MAIS plasmids pVT2 and pLR7 was measured to determine if plasmids from environmental and clinical isolates were related. Plasmid pVT2 hybridized under stringent conditions with 13.5 kb and 15.3 kb plasmids from both clinical and environmental MAIS isolates. Similarly, environmental and non-AIDS clinical MAIS strains contained plasmids from 15.3 to 25 kb related to pLR7. Earlier, Crawford and Bates showed that MAIS strains isolated from AIDS patients contained a small plasmid related to pLR7 (Crawford and Bates, 1986).

Plasmid pVT2 hybridized with all plasmids of 13.5 kb or smaller and with four plasmids of 15.3 kb, while the probe prepared from pLR7 hybridized with plasmids from 15.3 to 25 kb. Thus we can define, at present, three classes of small (less than 26 kb) plasmids in MAIS: Group 1, pVT2-related plasmids; Group 2, pLR7-related plasmids; and Group 3, plasmids not closely related to either probe. Since plasmids are placed in Group 3 simply by default, it is possible that this group could be subdivided further as new probes are employed. The weakly pVT2-hybridizing 28 kb plasmids in strains LR25 and MD17 presumably share shorter or less closely related regions with pVT2 than do the Group 1 plasmids.

Masaki and colleagues (1989) used plasmid pSS22 (20 kb), from a MAIS strain isolated from swine, as a DNA probe. Plasmid pSS22 hybridized with plasmids ranging in size from 17 to 20 kb from four porcine and two human MAIS isolates. It remains to be shown what relationship this plasmid probe

shares with other MAIS plasmids, but the results of our study suggest that the pSS22-related plasmids should prove to be Group 2, or possibly, group 3 plasmids, but not Group 1. The answer to this question could aid our understanding of the epidemiology of MAIS among swine and humans in relation to strains in the environment.

SalI digests of pVT2 and of one 13.5 kb and one 15.3 kb pVT2-related plasmid yielded several restriction fragments that were shared among the plasmids (Figure 4). Further, pVT2 hybridized with all but the smallest fragment resulting from the SalI digests (Figure 4), demonstrating that pVT2 shares large regions of sequence identity with these representative 13.5 and 15.3 kb plasmids. The pLR7-related 15.3 kb plasmids, although similar in migration rate on agarose gels, are clearly not closely related to the pVT2-related 15.3 kb plasmids, based on hybridization and restriction analysis (Figures 2 and 4).

The results of this work are of significance to our understanding of the epidemiology and virulence of MAIS. First, the results indicate that the clinical and environmental pools of MAIS are not genetically isolated, and thus support the contention that the source of human MAIS infection is the environment (Fry, et al., 1986; Meissner and Falkinham, 1986). This assertion is reinforced by the observation that the 15.3 kb pVT2-related plasmids were found only in soil and clinical isolates from Westchester County, New York (Tables 1 and 2). Second, pVT2- or pLR7-related plasmids were found more frequently in clinical isolates than in plasmid-bearing environmental isolates, and more frequently in aerosol (3 of 3, 100%) than in water (1 of 7, 14%) isolates (Table 2). This suggests that only a fraction of environmental isolates are potentially pathogenic, and that this fraction is enriched in aerosol

TABLE 2

Distribution of plasmid homology groups among plasmid-bearing MAIS isolates.

Strain Origin	Total	Number (%) of strains bearing plasmids related to:			
	Strains	pVT2	pLR7	Both	Neither

Total isolates	39	22 (56)	16 (41)	10 (26)	11 (28)
Total clinical isolates	23	15 (65)	12 (52)	8 (35)	4 (17)
non-AIDS isolates	17	10 (59)	6 (35)	3 (18)	4 (24)
AIDS isolates	6	5 (83)	6 (100)	5 (83)	0 (0)
Environmental isolates	16	7 (44)	4 (25)	2 (12)	7 (44)

isolates. That pVT2- and pLR7-related plasmids occur more frequently in strains isolated from AIDS patients than in clinical non-AIDS isolates is consistent with the observation of Horsburgh and coworkers (1986) that AIDS and non-AIDS clinical isolates differ. Finally, the fact that pLR7-related (Table 2) (Crawford and Bates, 1986) or pVT2-related (Table 2) plasmids are found singly in clinical isolates raises the possibility that both might encode virulence functions, as suggested by Crawford and Bates (1986). If that is so, it raises the question of whether plasmids in each of the three (at least) distinct small plasmid types (Table 2, Figure 2) encode different virulence functions. In addition, these cloned plasmids may be used as DNA probes to identify potentially pathogenic MAIS strains in culture or patient specimens.

Finally, the fact that larger, representative plasmids of groups 1 and 2 shared common restriction fragments with the plasmid probes pVT2 and pLR7, respectively (Figure 4) (Crawford and Bates, 1986), suggests that the additional DNA in those larger group 1 and 2 plasmids may be inserted at a unique insertion site in the fragments which differ in size. Thus our attention will be focused not only on the possible role of pVT2 and pLR7 in virulence as suggested by Crawford and Bates (1986), but also on the identification of the putative integration sites and the sequence of the inserted DNA.

CHAPTER 3

IDENTIFICATION OF TWO ADDITIONAL PLASMID HOMOLOGY GROUPS IN MYCOBACTERIUM AVIUM, M. INTRACELLULARE, AND M. SCROFULACEUM

Submitted to Plasmid.

INTRODUCTION

Members of the Mycobacterium avium, M. intracellulare, and M. scrofulaceum group (MAIS) are opportunistic human pathogens (Wolinsky, 1979). Disseminated MAIS infections are the most common systemic bacterial infection in U.S. AIDS patients (Ellner, 1991), being reported in 25% of AIDS patients (Blaser and Cohn, 1986). Further, pulmonary MAIS infections in the non-AIDS population may be increasing in prevalence (Ellner, 1991). Studies indicate that the environment is a source of human infection (Wolinsky, 1979; Falkinham et al., 1980) based on the identity of clinical and aerosol isolates (Fry et al., 1986; Meissner and Falkinham, 1986). Though available data support the hypothesis that the source of MAIS infection is MAIS organisms in the environment, this has not been proven. This is due, in part, to the absence of useful epidemiological markers for MAIS. Because almost all MAIS isolates from AIDS patients carry a variety of small plasmids (Crawford and Bates, 1986; Jucker and Falkinham, 1990), we have sought to identify related plasmids in order to use them as epidemiological markers.

Crawford and Bates (1986) showed that a DNA probe prepared from a plasmid (pLR7) from a clinical M. avium strain hybridized with small (less than 25 kb) plasmids of strains isolated from AIDS patients. We have reported the use of two cloned MAIS plasmids, pLR7 and pVT2, as DNA probes to show that each of these probes hybridized with a distinct set of small plasmids, thus defining two homology groups among MAIS plasmids (Jucker and Falkinham, 1990). Further, both these probes hybridized with plasmids from MAIS isolates recovered from environmental samples and from AIDS and

non-AIDS patients. However, many of the small plasmids tested did not hybridize with either pVT2 or pLR7.

In this report we describe the use of two MAIS plasmids from clinical isolates, a 16.3 kb plasmid from strain SC2 and a 17.4 kb plasmid from strain VA2, as novel DNA probes. Each of these probes hybridized with a distinct set of plasmids, thus demonstrating third and fourth homology groups among MAIS plasmids.

METHODS

The 22 plasmid-bearing MAIS strains used in this study are listed in Table 3. Strains 3802, 6651, and 7024 were isolated from blood or bowel tissue of AIDS patients at Dartmouth-Hitchcock Medical Center, New Jersey and strain D2-28 was isolated from a hot water sample collected from the Dartmouth-Hitchcock Medical Center.

Plasmid DNA was isolated from mycobacteria using D-cycloserine and ampicillin treatment before alkaline lysis (Jucker and Falkinham, 1989). Alkaline transfer of plasmid DNA from agarose gels to Zeta Probe^R membranes were performed according to Bio-Rad instructions. Hybridizations were conducted at 50°C in 50% formamide, 4X SSPE 1% SDS, and 0.5% dried skim milk powder (Sambrook, et al., 1989).

The 16.3 kb plasmid (apparent molecular weight of circular plasmid DNA) from strain SC2 and the 17.4 kb plasmid from strain VA2 were prepared for use as DNA probes as follows. Crude plasmid DNA was obtained as described above and extracted with phenol-salt (Kado and Liu, 1981) and phenol-chloroform (Sambrook et al., 1989). Electrophoresis of the lysate was performed in a 0.7% Seaplaque GTG low melting point agarose gel (FMC Corp., Rockland, ME) and the appropriate plasmid bands were cut out of the gel (Sambrook, et al., 1989). The gel chunks were dialyzed by immersing in about 50 volumes of water on ice for 30 minutes. Restriction endonuclease digestion was performed in the gel according to the manufacturer's instructions (FMC Corp., Rockland, ME). Both plasmids contained single PstI sites, while the 17 kb plasmid from VA2 also contained a single EcoRI site. The plasmids, linearized with PstI, were loaded on a second low melting point

Table 3. DNA homologies and apparent molecular weights of undigested plasmids recovered from clinical and environmental MAIS isolates.

Strain	Source	Plasmids (kb)	Reference
Human			
LR25	Non-AIDS	17.0 ² 28 162	Crawford et al., 1981
LR113	Non-AIDS	15.3 ²	Crawford and Bates, 1984
MD1	Non-AIDS	12.9 ¹	Meissner and Falkinham, 1986
MD2	Non-AIDS	21 44	"
MD17	Non-AIDS	17.5 18.5 28	"
MD22	Non-AIDS	15.3 ¹ 19.0 ⁴ 24 ² 160	"
SC2	Non-AIDS	16.3 ³ 31	"
SC7	Non-AIDS	13.5 ¹ 17.6 25 29 160	"
VA2	Non-AIDS	13.5 ¹ 17.4 ⁴ 24 35 54 135	"
VA3	Non-AIDS	13.5 ¹ 17.0 ² 155	"
VA10	Non-AIDS	13.5 ¹ 16.4 24 62 155 230	"
VA14	Non-AIDS	15.6 28 58 240	"
3802	AIDS	15.5	this study
6651	AIDS	17.6 23 28 140	this study
7024	AIDS	15.3 17.4	this study

¹ Hybridized with pVT2 (Jucker and Falkinham, 1990).

² Hybridized with pLR7 (Jucker and Falkinham, 1990).

³ Hybridized with pVT30, the 16.3 kb plasmid from SC2.

⁴ Hybridized with pVT40, the 17.4 kb plasmid from VA2.

Table 3, continued.

Strain	Source	Plasmids (kb)	Reference
Environmental			
CL26	chicken litter	13.5 ¹ 17.7 ³ 22 24 26 170	Falkinham et al., 1989
CL28	chicken litter	13.5 ¹ 15.5 160	"
D2-28	hospital	20 115	this study
SR353	water	23 ³	Falkinham et al., 1980
W39	water	19.5	"
W258	water	21	"
W1233	soil	15.3 ¹	Jucker and Falkinham, 1990

¹ Hybridized with pVT2 (Jucker and Falkinham, 1990).

² Hybridized with pLR7 (Jucker and Falkinham, 1990).

³ Hybridized with pVT30, the 16.3 kb plasmid from SC2.

⁴ Hybridized with pVT40, the 17.4 kb plasmid from VA2.

agarose gel using urea sample buffer (FMC, Rockland, ME) to inhibit regelling of samples in the well. The appropriate DNA bands were again cut out, dialyzed as above, and radiolabeled in gel with ^{35}S dCTP (NEN duPont, Cambridge, MA) according to FMC instructions using a random primer labeling kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Approximately 10 ng of probe with a specific activity of 5×10^5 dpm/ng for each plasmid was obtained per reaction by this technique and the probes were used at a concentration of approximately 0.5 ng DNA/ml of hybridization mixture.

RESULTS

The DNA probe prepared from the 16.3 kb plasmid from strain SC2 hybridized under stringent conditions with a 17.7 kb plasmid from environmental isolate CL26 and with a 22.5 kb plasmid from environmental isolate SR353 (Figure 5, Table 3). The 17.4 kb plasmid from clinical isolate VA2 hybridized only with a 16.9 kb plasmid from clinical isolate MD22 (Figure 5, Table 3). The probes did not hybridize with each other or with a plasmid which hybridized with the other probe. Neither probe hybridized with a plasmid which is related to pVT2 or to pLR7. Thus, a third plasmid homology group, defined by the 16.3 kb plasmid pVT30 from strain SC2 and a fourth plasmid homology group, defined by the 17.4 kb plasmid pVT40 from strain VA2 can be assigned.

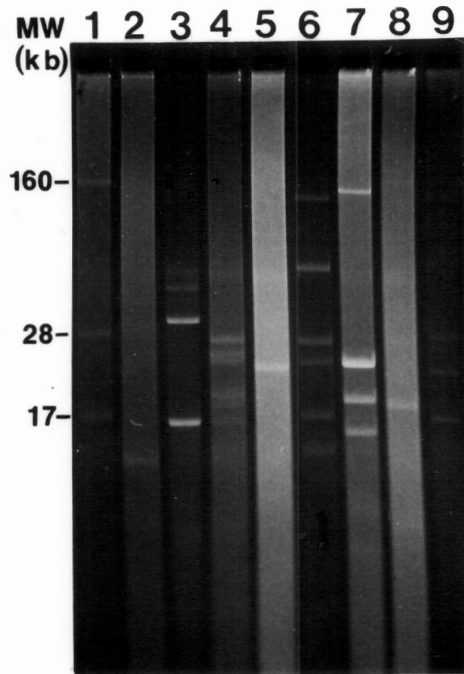


Figure 5A. Agarose gel electrophoresis of undigested plasmid DNA prepared from MAIS isolates. Lane 1, strain LR25, which contains plasmids of 162, 28, and 17 kb, from top to bottom; Lane 2, MD1; Lane 3, strain SC2, containing pVT30; Lane 4, CL26; Lane 5, SR353; Lane 6, strain VA2, containing pVT40; Lane 7, MD22; Lane 8, W39; Lane 9, 6659.

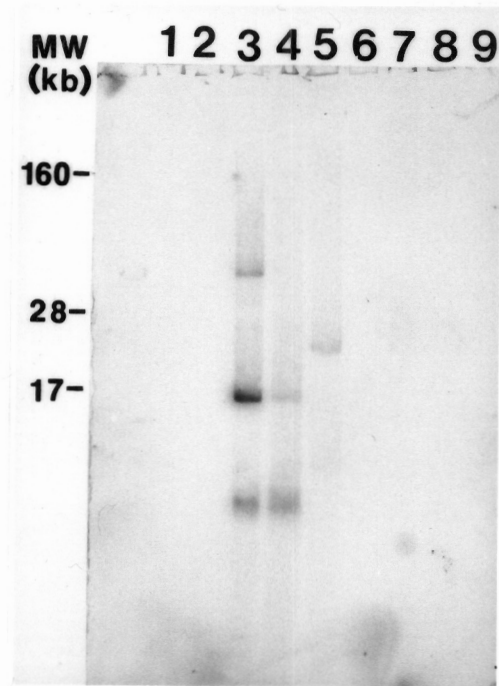


Figure 5B. Autoradiograph of a Southern transfer of gel 5A, which was hybridized with radiolabeled pVT30 DNA.

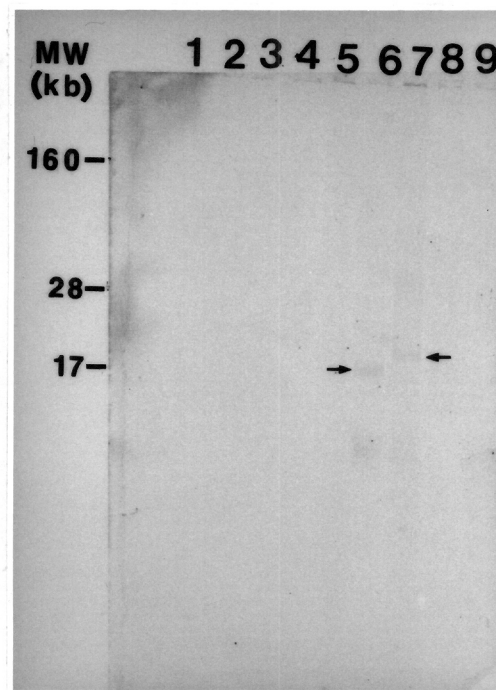


Figure 5C. Autoradiograph of a Southern transfer of a gel identical to 5A, which was hybridized with radiolabeled pVT40 DNA. The bands in lanes 6 and 7, indicated by arrows, are faint.

DISCUSSION

A method for restriction digestion and radiolabeling mycobacterial plasmids directly in low melting point agarose was adapted. This method allowed the use of plasmids from strains with multiple plasmids as radio-labeled DNA probes without cloning. Thus the method might be advantageous when the objective is a preliminary survey of sequence similarities with a new plasmid DNA probe.

The observation that plasmids from strains isolated from patients hybridized with plasmids from environmental isolates confirms earlier results (Jucker and Falkinham, 1990) and reinforces the hypothesis that environmental strains are a source of human infection.

Plasmids related to the 16.3 kb plasmid pVT30 from strain SC2 (Group 3) were found in three of 22 strains (14%) and plasmids related to the 17.4 kb plasmid pVT40 from strain VA2 (Group 4) were found in two of 22 strains (9%; Table 3, Figure 5). This indicates that plasmids related to either of the two probes occur relatively rarely, in contrast to plasmids which are related to pVT2 (Group 1) or to pLR7 (Group 2), which were found in 56% and 41%, respectively, of total isolates examined earlier (Jucker and Falkinham, 1990). Further, no Group 3 or Group 4 plasmids were found among AIDS isolates. The potential utility of the 16.3 kb plasmid from strain SC2 and the 17.0 kb plasmid from strain VA2 might lie in the scarcity of plasmids in Group 3 or Group 4, providing a more specific epidemiological marker than pVT2 or pLR7.

Although four homology groups have now been established for MAIS plasmids, many small MAIS plasmids which have been examined do not hybridize with any one of the four probes which have been used. Thus, there must be at least five MAIS plasmid homology groups, and possibly several many more. These results indicate that there is great diversity among MAIS plasmids.

CHAPTER 4

CONSERVED DNA SEQUENCES IN PLASMIDS OF MYCOBACTERIUM AVIUM, M. INTRACELLULARE, AND M. SCROFULACEUM

To be submitted to Plasmid.

INTRODUCTION

Members of the Mycobacterium avium, M. intracellulare, and M. scrofulaceum group (MAIS) are significant opportunistic human pathogens (Wolinsky, 1979). Disseminated MAIS infections are reported in 25% of AIDS patients (Blaser and Cohn, 1986) and are considered the most common systemic bacterial infection in U.S. AIDS patients (Ellner, 1991). In addition, the prevalence of MAIS infection may be increasing in the rest of the population (Ellner, 1991). Studies indicated that the environment is a source of human infection (Wolinsky, 1979; Falkinham, et al., 1980), based on the identity of clinical and aerosol isolates (Fry, et al., 1986; Meissner and Falkinham, 1986; Jucker and Falkinham, 1990)

Plasmids are common in MAIS (Crawford and Bates, 1984; Meissner and Falkinham, 1986). Although possible mechanisms for the relationship of plasmids and virulence remain unclear, 65% of clinical MAIS isolates have plasmids (Meissner and Falkinham, 1986). Further, almost all isolates from AIDS patients carry a variety of small plasmids (Crawford and Bates, 1986; Jucker and Falkinham, 1990). Plasmids have been shown to encode restriction-modification (Crawford, et al., 1981) and functions involved in mercury (Meissner and Falkinham, 1986) and copper resistance (Erardi, et al., 1987), but no practical selectable markers have been established for MAIS.

Four homology groups of small (< 25 kb) MAIS plasmids have been defined, based on hybridization with probes developed from MAIS plasmids (Crawford and Bates, 1986; Jucker and Falkinham, 1990; Jucker and Falkinham, submitted to Plasmid). Group 1 is composed of plasmids from 12.9 kb to 15.3 kb which hybridize with pVT2, a plasmid from a clinical non-AIDS

isolate MD1. Group 2 includes plasmids from 15.3 kb to 24 kb which hybridize with pLR7, a plasmid from clinical non-AIDS isolate LR113 (Crawford and Bates, 1984).

We sought to identify the nature of conserved DNA sequences in Group 1 plasmids because of the possible role of plasmids in virulence and interest in locating non-conserved regions as potential insertion sites of genetic markers, and identifying conserved plasmid regions which may encode essential replication functions for use in MAIS cloning vectors.

This study shows that representative Group 1 plasmids from 14 MAIS strains appeared to share significant regions of conserved DNA, based on restriction fragment conservation and hybridization. Restriction maps were developed for two Group 1 plasmids, pVT2 (12.9 kb and pVT4 (15.3 kb), that showed there are at least two conserved regions in the two plasmids, separated by non-conserved DNA. The DNA sequences of representative 435-base-pair DNA fragments of conserved regions from each plasmid appeared identical.

METHODS

Bacteria. MAIS strains used in this study are listed in Table 4.

Plasmid Isolation. Mycobacteria were grown and treated with antibiotics for plasmid isolation as described earlier (Meissner and Falkinham, 1986). Bacteria harvested from 30 ml of culture (8,000 x g for 10 min at 4°C) were resuspended in 0.4 ml TE buffer (Sambrook, et al., 1989) containing 5 mg subtilopeptidase/ml (Sigma Chemical Co., St. Louis, MO; Patel, et al., 1986) and incubated at 37°C for 1h or until the viscosity of the suspension increased. Lysing solution (0.8 ml, Kado and Liu, 1981) was added and mixed by rotating the tubes. The tubes were heated to 60°C for 20 min and cooled at room temperature for 15 min. The mixture was extracted once with phenol-salt (Kado and Liu, 1981) and centrifuged at 12,000 x g for 30 min at 4°C. One-half volume of 7.5 M ammonium acetate was added to the aqueous supernatant transferred to a microfuge tubes. The mixture was incubated on ice for 15 min and centrifuged at 14,000 x g for 15 at 4°C. The supernatant was recovered and DNA precipitated with two volumes of ethanol (Sambrook, et al., 1989). The DNA-containing pellet was dissolved in 300 µl TE and treated at 43°C for 20 min with a mixture of RNase A and RNase T₁ as described by Sambrook, et al. (1989). Sodium dodecyl sulfate and proteinase K (Sigma Chemical, St. Louis, MO) were added to final concentrations of 0.5% and 0.1 mg/ml, respectively, and the mixture incubated at 43° for 30 min. The mixture was extracted with phenol-chloroform-isoamyl alcohol (Sambrook, et al., 1989) and total plasmid DNA precipitated as above.

DNA Restriction Mapping, Cloning and Hybridization. Total plasmid DNA was isolated as described above from 14 MAIS strains (MD1, CL25, DE20,

Table 4. MAIS strains and DNA homologies and apparent molecular weights of undigested plasmids recovered from clinical and environmental isolates.

Strain	Source	Plasmids (kb)	Reference
Human			
GA1	Non-AIDS	13.5 ¹ 22 42 33 155	Meissner and Falkinham, 1986
MD1	Non-AIDS	12.9 ¹	"
MD22	Non-AIDS	15.3 ¹ 19.0 ⁴ 24 ² 160	"
SC7	Non-AIDS	13.5 ¹ 17.6 25 29 160	"
VA2	Non-AIDS	13.5 ¹ 17.4 ⁴ 25 39 54 135	"
VA3	Non-AIDS	13.5 ¹ 17.0 ² 155	"
VA10	Non-AIDS	13.5 ¹ 16.4 24 62 155 230	"
542	AIDS	13.5 ¹ 19.5 ²	Crawford and Bates, 1986
2812	AIDS	15.3 ¹ 17.6 ²	Jucker and Falkinham, 1990
2816	AIDS	15.3 ¹ 19.3 ²	"
Environmental			
CL25	chicken litter	13.5 ¹ 160	Falkinham et al., 1989
DE20	water	13.5 ¹ 17.6 ² 160 180	Falkinham et al., 1980
W1158	soil	15.3 ¹ 21 ²	Jucker and Falkinham, 1990
W1233	soil	15.3 ¹	"

¹ Hybridized with pVT2 (Jucker and Falkinham, 1990).

² Hybridized with pLR7 (Jucker and Falkinham, 1990).

³ Hybridized with pVT30 (Chapter 3).

⁴ Hybridized with pVT40 (Chapter 3).

GA1, MD22, SC7, VA2, VA3, VA10, W1158, 542, 2812, 2816, and W1223; Table 4) that carry Group 1 plasmids, cut with *Sall*, and separated by electrophoresis on 1% agarose gels. DNA was transferred to Zeta-Probe membranes by alkaline blotting as described (Jucker and Falkinham, 1990). The 9.7 kb and 5.6 kb fragments of pVT4, cloned into pUC19 to form pVT111 and pVT110, respectively (Table 5), were excised from the vectors with *EcoRI* and *HindIII*, separated on low melting point agarose, and radiolabeled with ^{35}S -dCTP using the manufacturer's instructions (FMC, Rockland, ME). Hybridizations were conducted overnight at 50°C in 50% formamide, 4X SSPE (Sambrook, et al., 1989), 1% SDS, and 0.25 % skim milk powder.

Restriction digestions and restriction mapping of the two related plasmids pVT2 and pVT4 (Figure 6) were performed as described (Sambrook, et al., 1989). Fragments of the two plasmids were cloned to facilitate restriction mapping and DNA sequencing and for use as probes to identify the relationship of the conserved sequences. DNA fragments were ligated (Table 5) with pUC19 (Yannisch-Perron, et al., 1985) or with pBluescriptSK- II or pBluescriptSK+ II (Stratagene, CA) in Sea Plaque low melting point agarose (FMC Corp., Rockland, ME) as described by Sambrook (1989).

Thermal Stability Experiments. DNA sequence similarity was assessed by filter-bound thermal dissociation experiments (Johnson, 1981) using probes and target DNA from conserved region b (Figure 6) from pVT2 (cloned as pVT102; Table 5) and from pVT4 (cloned as pVT112; Table 5). To prepare target DNA, pVT102, pVT112, or non-related pVT101 DNA (Table 5) were linearized with an appropriate restriction enzyme, denatured in 0.5 N NaOH, and vacuum-filtered through 45 mm diameter Zeta-Probe discs (Bio-Rad, La Jolla, CA) according to manufacturer's instructions, at concentrations of $0.1 \mu\text{g}/\text{cm}^2$ or

Table 5. Recombinant plasmids constructed from fragments of pVT2 and pVT4. Restriction endonuclease site coordinates are given as shown in Figure 6.

Construct	Source	Fragment Cloned ¹	Insert Size (kb)	Vector	Subclone of
pVT100	pVT2	EcoRI,1 to HindIII,4.2	4.2	pUC19	pVT2
pVT110	pVT4	EcoRI,1 to HindIII,5.5	5.5	pUC19	pVT4
pVT101	pVT2	HindIII,4.2 to EcoRI,1	8.6	pUC19	pVT2
pVT111	pVT4	HindIII,5.5 to EcoRI,1	9.2	pUC19	pVT4
pVT102	pVT2	BglII,2.3 to HindIII,4.2	1.9	pBluescriptSK-II	pVT100
pVT112	pVT4	BglII,3.6 to HindIII,5.5	1.9	pBluescriptSK-II	pVT110
pVT104-	pVT2	BglII,2.3 to NotI,2.74	0.44	pBluescriptSK-II	pVT102
pVT104+	pVT2	BglII,2.3 to NotI,2.74	0.44	pBluescriptSK+II	pVT104-
pVT114-	pVT4	BglII,3.6 to NotI,4.04	0.44	pBluescriptSK-II	pVT112
pVT114+	pVT4	BglII,3.6 to NotI,4.04	0.44	pBluescriptSK+II	pVT114-

¹Restriction endonuclease sites are designated clockwise from the EcoRI site.

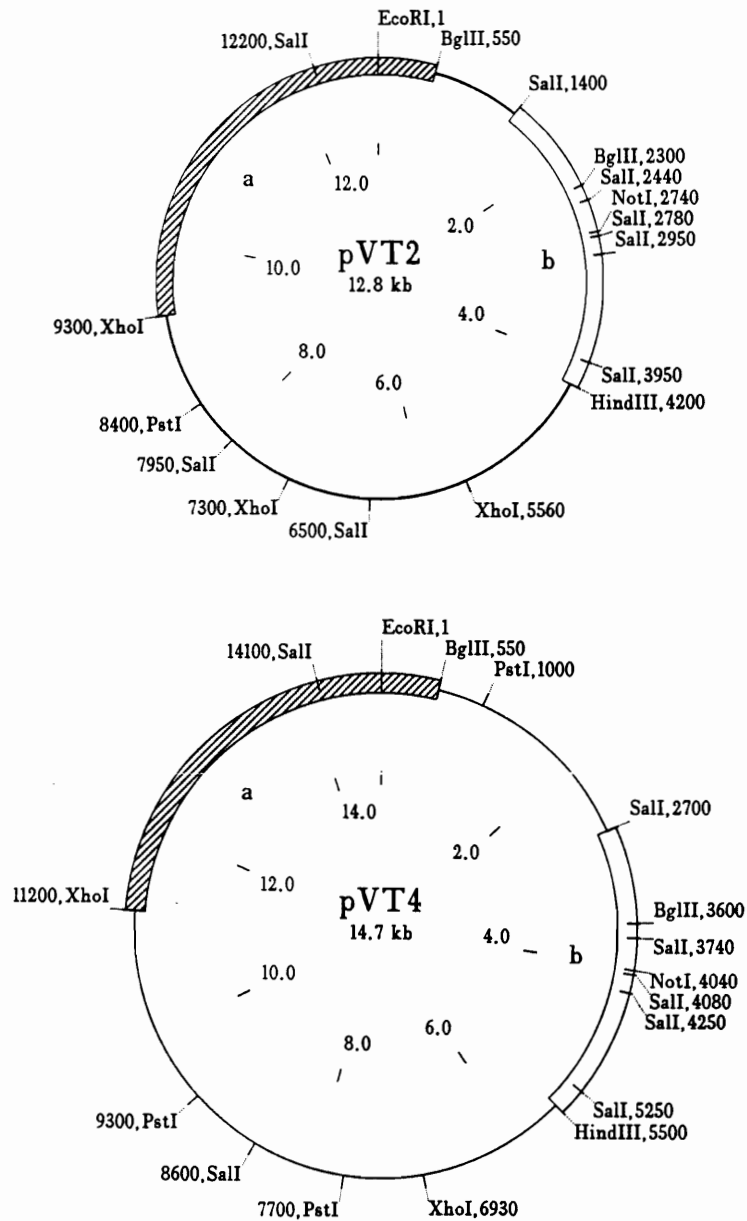


Figure 6. Restriction maps of plasmids pVT2 and pVT4. The restriction endonuclease site coordinates are in basepairs; the scales inside the circles are in kilobasepairs. Each boxed area, arbitrarily designated a or b, represents a region which appears to be conserved in both plasmids. NotI has only been mapped from BglII, 2300 to HindIII, 4200 in pVT2, and from BglII, 3600 to HindIII, 5500 in pVT4. There are no sites for XbaI or BamHI.

0.6 $\mu\text{g}/\text{cm}^2$ The filters were dried in a vacuum oven at 80° for 1h and 7 mm diameter discs were cut from them.

The cloned inserts of pVT102 and pVT112 (Table 4) were isolated and radiolabeled as described above. A modification of the protocol described by Johnson (1981) was used for filter dissociation experiments, as follows. Each probe was mixed with a hybridization membrane containing either self DNA, the analogous conserved DNA (region b) from the other plasmid, or with unrelated DNA pVT101. Hybridizations were conducted overnight at 50°C in 45% formamide, 4X SSPE, 1% SDS, and 0.5% skim milk powder. After hybridization the filter discs were rinsed in 2X SSPE, 0.1% SDS; 1X SSPE, 0.1% SDS; and 0.1X SSPE, 1% SDS, in sequence for 10 min each at room temperature. Each disc was placed in a 0.5 ml microfuge tube with 450 μl dissociation buffer (0.1X SSPE, 10% formamide, 1% SDS, 0.5% skim milk powder). The filters were then passed through a succession of baths of dissociation buffer in sealed tubes at temperatures increasing by 5°C intervals from 45°C to 90°C, with 5 min in each bath, in a Perkin-Elmer Cetus Thermal DNA Cycler. The amount of radioactivity eluted at each time point was measured by liquid scintillation counting.

DNA Sequencing. Single-stranded DNA for sequencing was generated from subclones of 435-bp conserved DNA regions from pVT2 (pVT104+, pVT104-) and from pVT4 (pVT114+, and pVT114-) cloned into pBluescriptSKII vectors (Table 5) using helper phage VCSM13 (Stratagene, La Jolla, CA) according to the manufacturer's protocol (Strategies 5 Vol. 3, Stratagene) in host Escherichia coli strain XL1-Blue (Bullock, et al., 1987). Single stranded DNA was sequenced with a Sequenase 2.0 kit (U.S. Biochemical, Cleveland, OH) according to the manufacturer's instructions. Labeling reactions were incubated at 20°C and termination reactions were incubated at 40°C. The DNA inserts were sequenced from both directions. Six % acrylamide buffer gradient sequencing gels were run according to the manufacturer's instructions (IBI, Indianapolis, IN).

The GenBank/EMBL and NBRF protein databanks (version 8/91) were searched for DNA sequences with similarities to the pVT104/114 sequence using the Sequence Analysis Software Package (Devereux, et al., 1984; Genetics Computer Group, Madison, WI).

RESULTS

Restriction Mapping. Total plasmid DNA isolated from 14 MAIS strains which carry Group 1 plasmids was subjected to SalI digestion and Southern analysis using pVT4 DNA as a probe. Figure 7 shows that restriction fragments of 0.4 kb (faint), 1.0 kb, and 1.05 kb were shared by the Group 1 plasmids of all strains except CL25. A 2.6 kb band was shared by plasmids from all strains except CL25 and W1233. A 5.5 kb fragment was shared by all strains except MD1 and CL25.

Restriction maps were developed for two related plasmids, pVT2, from MAIS strain MD1, and pVT4, from strain W1233 (Figure 6). Two distinct regions were shared between the two plasmids; designated regions a and b in Figure 6. The sizes of the plasmids in Figure 6 are based on apparent molecular weights of linear DNA fragments, and differ slightly from the apparent molecular weights of the undigested plasmids (Table 4).

Thermal Stability Experiments. DNA from conserved region b (Figure 6), shared by pVT2 (cloned as pVT102, Table 5) and pVT4 (cloned as pVT112, Table 5) was compared by thermal dissociation experiments on hybridization membranes. The radioactivity elution curves showed peaks at approximately 70°C under all conditions tried and with all permutations (i.e. pVT102 probe versus pVT102, pVT102 probe vs pVT112; pVT112 probe vs pVT112, or pVT112 probe versus pVT102). Results from a representative experiment are shown in Figure 8.

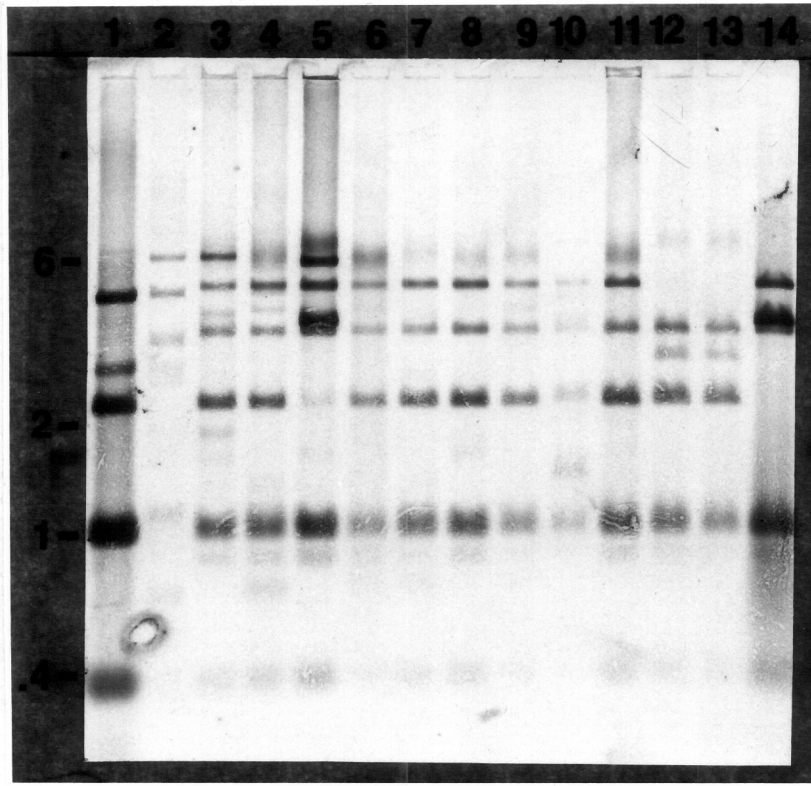


Figure 7. Autoradiograph of a Southern transfer of an agarose gel of *Sall* digests of plasmid DNA from MAIS strains which bear Group 1 plasmids. The DNA probe consisted of a mixture of the radiolabeled pVT4-derived inserts of plasmids pVT110 and pVT111. Lane 1, strain MD1, bearing pVT2; Lane 2, CL25; Lane 3, DE20; Lane 4, GA1; Lane 5, MD22; Lane 6, SC7; Lane 7, VA2; Lane 8, VA3; Lane 9, VA10; Lane 10, W1158; Lane 11, 542; Lane 12, 2812; Lane 13, 2816; Lane 14, W1233, bearing pVT4. The scale on the left indicates molecular weight in kilobasepairs.

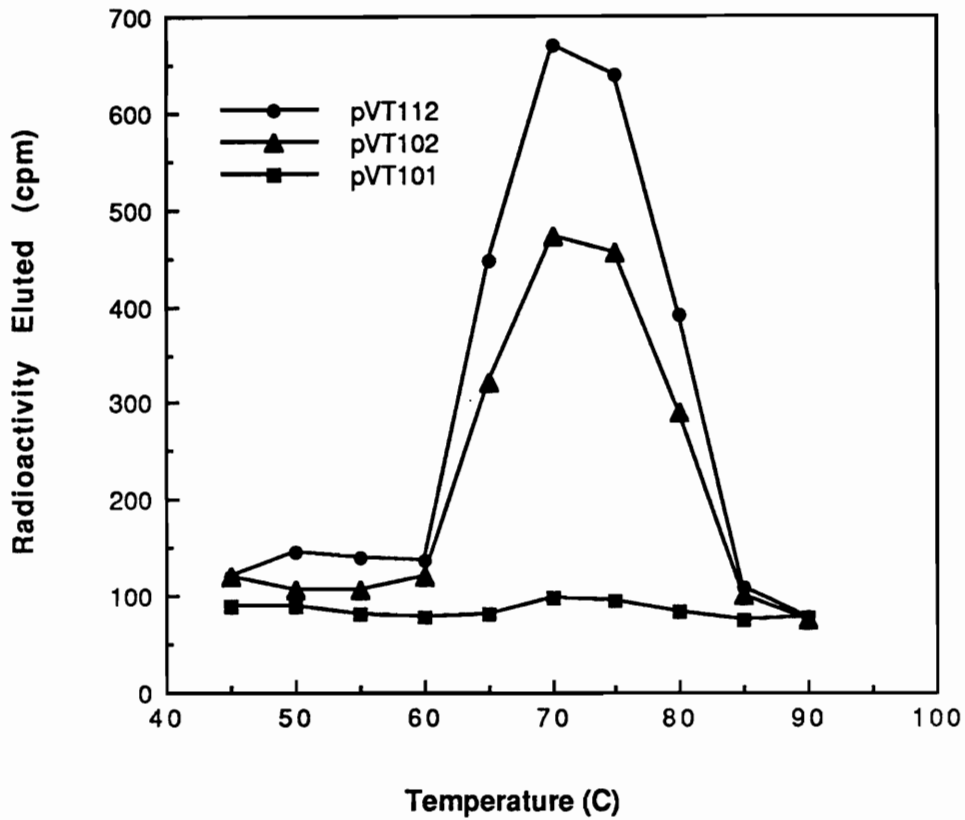


Figure 8. Thermal denaturation experiment with DNA from conserved region b (Figure 6) of plasmids pVT2 and pVT4. The radiolabeled pVT2-derived insert of pVT102 (Table 5) was hybridized with filter-bound DNA of either pVT102, pVT112 (derived from region b of pVT4; Table 5), or pVT101 (non-related DNA derived from pVT2; Table 5). Radioactivity was eluted from the filters in a series of baths of increasing temperatures.

DNA Sequencing. The 435-bp sequences of segments of region b (Figure 6) cloned in pVT104 (derived from pVT2; Table 4) and pVT114 (derived from pVT4; Table 4) are shown in Figure 9. No difference in the sequences of the two fragments was detected. A computer search of the GenBank/EMBL and NBRF protein databanks revealed no significant DNA sequence or potential amino acid sequence similarities. An uncharacterized open reading frame 350 bases long was found beginning at base 85 to the end of both inserts.

```

1  AGATCTGCGC CCGCACATCT CGGCGTCCAC AAGTCCAAC ACGGCCTGCT CTTCGTGGAG
   TCTAGACGCG GCGGTGTAGA GCCGAGGTG TTCCAGGTTG TGCCGGACGA GAAGCACCTC

61  GAACACCCCG AGGTTGAAGC GTTCATGGCC →ORF CTCGCGTTGA GCGCGGGCCC GCGCGGTGAC
   CTTGTGGGCG TCCCACTTCG CAAGTACCG GAGCGCAACT CCGCGCCGGG CGCGCCACTG

121 CCGCATTTCC AGCGCGTCGA CGGCGGCTC GACCATCTCC CGAGGCGAGC GTCGACATC
   GCGTAAGG TCGGCAGCT GCCCGCGAG CTGGTAGAGG GTCCTGCTCG CGAGCTGTAG

181 CACGGGCAGC TCGCGGCCA TCAACTCCAC CAGATCCGCG CGGTGAACG TCGCCTTGTC
   GTGCCCGTCG ACGCGGGGT AGTTAGGTG GTCTAGGCGC GCCACTTGC AGCGGAACAG

241 CATCGCCTCG GCCGCCCAAC AACCGCCGC GATCAAAAGC GCGTGGCCGC CGACCCAC
   GTAGCGGAGC CGCGGGTTG TTGGCGGCGG CTAGTTTTCG CGACCCGCG GCGTGGGTG

301 GCCCGGCCT TCGCGCCTC CTGGAAGCGT TCGCGATCAA ACCGTACACC CCGGCATCCG
   CGGCGCGGA ACGCGGGAG GACCTTCGA AGCGTAGTT TGGCATGTGG GGCCGTAGGC

361 CTTGCCACAT CGCGCCAGT TGTGCCCCAG CCAGTTGTTT GGGTTTGGTC GGGCGGTGG
   GAACGGTGTG GCGCGGTCA ACACGGGTC GGTCAACAAG CCCAAACCAAG CCCGCCACC

421 CCTTTTGGG GCGCG
   GGAAACCCG CCGGC

```

Figure 9. DNA sequence of the inserts of pVT104 and pVT114 (Table 5), containing portions of region b (Figure 8) of pVT2 and pVT4, respectively. An unidentified open reading frame extends from base 85 to base 435.

DISCUSSION

Plasmid Isolation. Exposure of Mycobacterium avium, M. intracellulare, and M. scrofulaceum (MAIS) cells to subtilisin before lysis was adopted from a genomic DNA preparation technique (Patel, et al., 1986) and increased plasmid DNA yields and provided more consistent lysis with strains of diverse growth characteristics (L.E. Via, personal communication). It appears that it is advantageous to include this step when lysing MAIS strains with average or slow growth rates.

Plasmid DNA preparations were treated with proteinase K (Patel, et al., 1986) before restriction digestion. This treatment reduced the requirement for extensive organic extraction or other treatments (e.g., GeneClean glass milk, before MAIS plasmid DNA can be efficiently cut with restriction endonucleases. Proteinase K-treated preparations showed fewer partial restriction digestion fragments and less smearing and adherence of DNA to wells in agarose gels. Other advantages of proteinase K treatment include the shorter time required and the recovery of more DNA.

Even with those treatments, partial restriction digest bands are visible in some lanes in Figure 7. This result implies that an uncharacterized, restriction enzyme-inhibiting substance adhered tightly to MAIS plasmid DNA despite phenol-chloroform extractions, proteinase treatment, and ethanol precipitation in the presence of ammonium acetate.

Restriction Analysis. SalI digestion of plasmid DNA from 14 Group 1 plasmid-bearing MAIS strains and Southern analysis with a DNA probe derived from pVT4 showed that a significant number of restriction fragments were shared. The similarity of restriction fragments under conditions of high

stringency supports the hypothesis that these representative Group 1 plasmids share extensive regions of conserved DNA.

Seven different restriction patterns can be discerned in Figure 7, although all lanes share bands. The 12.9 kb plasmid, pVT2, shows unique pattern, as does the 13.5 kb plasmid from strain CL25. The 13.5 kb plasmids from strains DE20, GA1, SC7, VA2, VA3, and VA10 share a third restriction pattern. The five 15.3 kb Group 1 plasmids from strains W1158, 542, 2812, 2816, and W1233 (pVT4) each show unique restriction patterns except strains 2812 and 2816. Strikingly, strains 2812 and 2816 were isolated from two different AIDS patients at Westchester County (NY) Jail. The results imply that, although the Group 1 plasmids are closely related, they have undergone a series of insertion and/or deletion events.

Interestingly, the Group 1 plasmid from clinical non-AIDS isolate MD22 shows the same pattern as pVT4, a 15.3 kb plasmid from soil isolate W1233. The Group 1 plasmid from strain MD22 had initially been found with a size of 13.5 kb (P.S. Meissner, dissertation; Jucker and Falkinham, 1990), but recently strain MD22 appears to have a 15.3 kb Group 1 plasmid (Table 4). Possibly the strain MD22 Group 1 plasmid has experienced a recent insertion event. Further, it may be inferred, at least in preliminary this example, that the transition from a 13.5 kb to a 15.3 kb Group 1 plasmid may be a discrete event. Further experiments may isolate an IS-like element(s) from MAIS (see also discussion below).

The 14 MAIS strains investigated (Table 4, Figure 7) include isolates from water (DE20), soil (W1158, W1233), chicken litter (CL25), and patients with (542, 2812, 1816) or without AIDS (GA1, MD22, SC7, VA2, VA3, VA10). The close relationship of the plasmids from environmental and clinical

isolates reinforces the assertion that environmental strains are not genetically isolated from clinical strains and that the environment is a source of human MAIS infection (Wolinsky, et al., 1979; Fry, et al., 1986; Meissner and Falkinham, 1986; Jucker and Falkinham, 1990).

Restriction Mapping of pVT2 and pVT4. Molecular cloning, restriction mapping, and hybridization experiments of Group 1 plasmids pVT2 (12.9 kb) and pVT4 (15.3 kb) showed that at least two distinct regions are conserved in the two plasmids (Figure 6). The regions are arbitrarily named conserved regions a and b and are separated by regions of non-conserved DNA. The distance between regions a and b (clockwise from the EcoRI site) is 1.3 kb greater in pVT4 than in pVT2. The distance between regions b and a (clockwise from the EcoRI site) is 0.6 kb greater in pVT4 than in pVT2. Thus pVT2 and pVT4 appear to be the products of several insertion and/or deletion events, and together with the other Group 1 plasmids, present interesting examples of plasmid evolution. Perhaps one or more of the conserved regions contain DNA essential to plasmid replication that might be exploited for the construction of a MAIS cloning vector.

Further restriction mapping and sequencing of pVT2 and pVT4 might result in the isolation of a site of DNA insertion, as mentioned above. One example of a likely position to begin such investigation would be clockwise from region a (Figure 6). A PstI site which is absent in pVT2 is located 0.45 kb from the BglII site at 0.55 kb in pVT4, indicating that the DNA within that 0.45 kb region is not conserved.

Thermal Stability Experiments. Thermal stability experiments were conducted with conserved region b (Figure 6) from pVT2 (cloned as pVT102; Table 5) and from pVT4 (cloned as pVT114; Table 5). No significant difference in T_M could be detected between the two DNA inserts and the probe from pVT102 (Figure 8), or the probe from pVT112 (data not shown), indicating that the DNA sequence of the two was highly conserved.

DNA Sequencing. When DNA sequencing was attempted with double-stranded pVT104 template the large number of stops (bands in all four lanes) on the sequencing gel precluded the reading of useful sequence information. Presumably the high G+C% in the sequenced DNA (70%) facilitates a significant amount of secondary structure and/or rapid renaturation of template DNA. It appears that sequencing with double-stranded templates is of little use in MAIS.

The DNA sequences of the inserts of pVT104 and pVT114, cloned from conserved region b of pVT2 and pVT4, respectively (Figure 6, Table 5), were identical (Figure 9). This complete sequence identity reinforces the close relationship of plasmids pVT2 and pVT4, and suggests that the sequenced regions of both plasmids originated from the same element a relatively short time ago.

CHAPTER 5

SUMMARY

The first objective of this study was to demonstrate that plasmids borne by clinical isolates of Mycobacterium avium, M. intracellulare, and M. scrofulaceum (MAIS) are closely related to plasmids from environmental MAIS isolates. Also, MAIS plasmid DNA probes were developed and used to show that MAIS plasmids could be placed into more than four groups based on hybridization. A second major objective was to determine if conserved DNA was shared among related plasmids of different sizes in homology Group 1. It was found that the Group 1 plasmids shared two regions of conserved DNA, interspersed with nonhomologous DNA. These results filled a gap in our basic knowledge of MAIS plasmid biology and are of significance to our understanding of MAIS epidemiology, and thus have implications on public health measures.

The hypothesis that the environment is a source of human MAIS infection was supported by epidemiological data, as discussed in Chapter 1, but remains a point of debate. When this study was initiated, Meissner and Falkinham (1986) had observed plasmids of similar size in MAIS from both clinical and environmental sources. However, there was no proof that these plasmids were closely related. The results of this work support the hypothesis that the environment is a source of human MAIS infection. This is borne out by the observation that MAIS plasmid DNA probes belonging to three different homology groups hybridized with plasmids from both clinical and environmental isolates. Hybridization experiments with restriction fragments of Group 1 plasmids provided additional evidence in support of the conclusion. Further, the fact that a 435-base conserved DNA sequence was identical in plasmids from a clinical isolate (MD1) and from a soil isolate

(W1233) implies a relatively recent transmission of genetic information from the environment to a human.

Four plasmid homology groups were identified, making available a novel and potentially valuable epidemiological tool. For example, Burns et al. (1991) used plasmid profiles to demonstrate that M. fortuitum isolates from 16 alcoholic patients were identical, and that the strain originated from a hospital water supply. These results agreed with phenotypic analysis and pulsed-field electrophoresis of large genomic DNA restriction fragments. The use of plasmid DNA probes in this type of investigation would give a greater degree of confidence in the results, and would eliminate potential questions arising from the occurrence of unrelated plasmids of similar size. In this study, five MAIS strains were found to have a 15.3 kb plasmid (Table 1). The 15.3 kb plasmid from strain LR113 appeared identical in size on agarose gels, but proved to be a Group 2 plasmid, unrelated to the 15.3 kb Group 1 plasmids in strains W1158, W1233, 2812, and 2816. Strikingly, strains W1158 and W1233 were isolated from soil in Westchester County, NY, while strains 2812 and 2816 were isolated from AIDS patients in Westchester County (NY) Jail.

Group 1 plasmids of 15.3 kb were not isolated from any other location. Assuming that the AIDS patients were not lifetime residents near Westchester County, this observation suggests that the AIDS patients in Westchester County Jail were infected relatively recently with local mycobacteria, and that the infecting strains were not harbored asymptotically over the long term until onset of AIDS symptoms allowed the growth of the bacteria. Such examples illustrate the potential utility of MAIS plasmid homology groups. It is likely that the plasmids which remain uncharacterized

can also be placed into homology groups by the development of additional plasmid DNA probes. Because most MAIS isolates from AIDS patients bear plasmids, and if most of the uncharacterized plasmids can be placed into additional homology groups, plasmid typing should prove useful for epidemiological studies.

Knowledge of MAIS plasmid homology groups may shed light on important unanswered questions. Can correlations be found between virulence or other characteristics and certain plasmid groups? Related plasmids would be expected to share characteristics. Is there a geographical correlation, local and worldwide, with certain plasmid groups, as suggested by the Westchester County isolates discussed above? A comprehensive study of more isolates using the four defined probes, plus possibly other probes, could yield answers to these questions. However, conclusions will not be possible until more is known about MAIS virulence mechanisms, and until plasmids can be transferred into plasmid-free strains for direct comparison of possible plasmid-encoded characteristics (e.g. virulence).

MAIS plasmids were placed into four homology groups, yet many (54%) of the over 36 different plasmids which have been observed in MAIS did not hybridize with the MAIS plasmid DNA probes (Tables 1 and 3). The members of the fifth, default group probably represent one or more homology groups. Thus it can be concluded that there is a great deal of diversity among MAIS plasmids. The function of this heavy investment in plasmid DNA by MAIS (i.e. as much as 9% of total DNA) remains mostly unknown (see Chapter 1). Whatever their function, the four plasmid homology groups appear to be incompatibility groups. No two plasmids of the same homology group were observed in one strain, as discussed below. Group 1 and Group 2 plasmids

were commonly isolated from the same strains. Strain MD22 contains plasmids belonging in Group 1, 2, and 4, and strain VA2 contains a Group 1 and a Group 4 plasmid, directly demonstrating that plasmids from Groups 1, 2, and 4 can exist in the same cell. In strain CL26 a Group 3 plasmid is found with a Group 1 plasmid. There is no observation of a Group 3 plasmid coexisting with a Group 2 or 4 plasmid, but this is more likely due to the relative scarcity of Group 3 plasmids than to incompatibility.

Tinge and Curtiss (1990) cured Salmonella spp. of a specific virulence plasmid by transduction with a phage or by transformation with a plasmid containing a par region which had been inactivated by the insertion of a Km^r marker. Under kanamycin selection, the par region of the resident plasmid was replaced by homologous recombination with the introduced, inactivated par region. Since par function is essential for plasmid maintenance, the plasmid with the inactivated par was eliminated. These types of experiments could be adapted to demonstrate plasmid incompatibility groups in MAIS. More significantly they might provide a way to obtain cured derivatives of MAIS strains. However, knowledge of MAIS genetics remains primitive compared to Salmonella, and many fundamental problems would have to be overcome, notably how to transport DNA into MAIS by either transduction with a phagemid, as has been done with M. smegmatis (Snapper, et al., 1987) or transformation with a plasmid vector, as described with M. bovis BCG (Goto, et al., 1991).

Multiple bands often resulted when Southern analyses were performed with undigested plasmids and plasmid DNA probes; this occurred frequently when using old lysates or preparations that had undergone repeated purification steps. Plasmid DNA preparations presenting multiple bands with

one probe were cut with a restriction enzyme that cut at a single site. This was repeated with five Group 1 plasmids, and with pVT30 (Group 3) and pVT40 (Group 4). In all cases, only a single band hybridized with the probe, demonstrating that the multiple bands were due to topoisomeric forms of the same plasmids. Future workers should keep in mind that it has not been proven that every band appearing on gels of undigested plasmid DNA represents a unique plasmid in that MAIS strain, especially with plasmids not in one of the four homology groups.

When this research was begun, the literature reported the use of CsCl gradient centrifugation for the rapid preparation of MAIS plasmid DNA suitable for restriction digestion. A rapid technique for this purpose was adapted from existing protocols (Chapter 2) which eliminated the need for CsCl gradient centrifugation and resulted in a significant gain in convenience and economy. Also, a useful method was adapted for the preparation of plasmid DNA probes from strains with several plasmids (Chapter 3).

Restriction analysis and hybridization experiments showed that 14 Group 1 plasmids shared extensive regions of highly conserved DNA. Representative Group 1 plasmids pVT2 (12.9 kb) and pVT4 (15.3 kb) were found to share two distinct conserved DNA regions, which were separated by non-conserved DNA (Figure 6). It is reasonable to assume that the conserved regions encode biologically significant functions, including genes essential for plasmid replication, maintenance, and segregation. Thus, one or more of the conserved regions might provide the basis for the construction of a MAIS cloning vector. A major obstacle that must be overcome before construction of a shuttle vector is the development of a way to introduce plasmid DNA

into MAIS. Also, a practical antibiotic or other resistance gene would have to be incorporated into the vector to provide selection.

Of the 14 Group 1 plasmids which shared extensive regions of conserved DNA, 7 different restriction patterns were observed. A comparison of restriction maps of Group 1 plasmids pVT2 (12.9 kb) and pVT4 (15.3 kb) (Figure 6) showed that the two conserved regions were interspersed with non-shared regions. The distances between each of the conserved regions was greater in pVT4 than in pVT2. Thus, it appears that Group 1 plasmids are the products of several insertion and/or replacement events.

A Group 1 plasmid from strain MD22, which was initially observed with a size of 13.5 kb (P.S. Meissner, dissertation), recently appeared as a 15.3 kb plasmid (Table 4). This new plasmid presented a restriction pattern identical to that of pVT4. It can be inferred that the transition from 13.5 to 15.3 kb plasmid is a discrete event. Further investigation of this event and a comparison of the MD22 Group 1 plasmid before and after insertion might lead to the isolation of a novel MAIS mobile genetic element. This element would presumably be endemic to all Group 1 plasmids. The source of the DNA inserted in the Group 1 plasmid in strain MD22 is unknown (i.e. chromosomal or plasmid). Hybridization experiments using a pVT2 DNA probe revealed no signs of pVT2-related DNA in digests of genomic DNA from strains MD1 or SC2 (taking into account the bands attributable to pVT2). A useful experiment would be to use cloned pVT4 DNA probes to hybridize with genomic digests of strain MD22 carrying the 13.5 kb Group 1 plasmid and with the derivative strain carrying the 15.3 kb plasmid. The hypothesis would state that a band would be detected in the chromosomal digest of strain

MD22 with the 13.5 kb plasmid, and that this pVT4-related DNA would be linked to the 15.3 kb plasmid in the MD22 derivative with the larger plasmid.

There is some evidence that pVT4 is not exclusively the product of insertion events, but that deletions or replacements have also occurred in the evolution of this Group 1 plasmid. In pVT4 the distance between conserved regions b and a, in a clockwise direction, is only 0.6 kb greater than the distance between the analogous regions in pVT2 (Figure 6). However, there is a region of at least 1.6 kb between conserved regions b and a in pVT4 that differs in restriction pattern from the analogous region in pVT2. This observation cannot be explained by a simple insertion or deletion event. Understanding of the exact nature of the events in the evolution of Group 1 plasmids awaits further research. It can be hypothesized that the 13.5 kb Group 1 plasmids are closest to the "ancestral" Group 1 plasmid because they are the most common and they appear identical by restriction and hybridization analysis. In this hypothesis the smaller and larger Group 1 plasmids would have been derived from the 13.5 kb plasmids by uncharacterized events.

The results of this study also have implications on plasmid transmission between MAIS strains. Several sets of Group 1 plasmids appeared identical by restriction fragment and hybridization analysis (Figure 7). For example, the 13.5 kb plasmids from strains DE20, GA1, SC7, VA2, VA3, and VA10 shared restriction fragment patterns. Also, the 15.3 kb plasmids in AIDS isolates 2812 and 2816 shared the same restriction fragment pattern. Each of these independent isolates had a unique plasmid profile (Table 1), as well as differences in growth characteristics, pigmentation (unpublished observations), and with VA2 and VA10, differences in the patterns of restriction

digests of genomic DNA (L.E. Via, unpublished). Therefore these strains, which carry very similar, if not identical, plasmids, are members of different clonal lines. Based on this circumstantial evidence, I propose that a mechanism for plasmid transfer exists in MAIS. It would be interesting to learn how a large, highly charged molecule such as a MAIS plasmid penetrates the thick hydrophobic envelope of a mycobacterial cell. The use of restriction fragment length polymorphism (RFLP) analysis in parallel with plasmid hybridization could yield more information about the relationship of plasmids and chromosomal DNA (J.J. McFadden, personal communication), provided that RFLP probes with sufficient discrimination become available.

Plasmids appear to be widespread, diverse, and actively evolving phenomena in MAIS and it must be assumed that they are significant to members of the Mycobacterium avium group. This study sets the stage for further analysis of the function and origins of plasmids from environmental and clinical isolates of Mycobacterium.

APPENDIX

ATTEMPTS TO CLONE MERCURY RESISTANCE GENES

INTRODUCTION AND REVIEW OF LITERATURE

Mercury resistance is widespread in bacteria and has been found in most eubacterial genera (Summers, 1986), including the Mycobacterium avium, M. intracellulare, M. scrofulaceum group (MAIS) (Meissner and Falkinham, 1984). Two types of non-specific mercurial transformation have been described -- the production of hydrogen sulfide, which reacts with mercury to form insoluble HgS ; and the production of methyl cobalamin, which mediates the methylation of mercuric ion (Hg^{2+}) in a non-enzymatic reaction. The resulting methyl mercury compounds are volatile and rapidly diffuse from the microbial environment (Robinson and Tuovinen, 1984). Both these processes are considered gratuitous in that neither is a specific response to mercury (Summers, 1986). There is a specific type of reductive mercury resistance system which includes a mercuric reductase and a Hg^{2+} uptake system which is encoded by a mer operon and whose expression is induced in the presence of mercury compounds (Summers, 1986). Resistance provided by the action of mercuric reductase is termed narrow-spectrum resistance. Broad-spectrum resistance systems include in addition an organomercurial lyase which cleaves mercuric ion from organomercurials such as methylmercury or phenylmercuric acetate (Robinson and Tuovinen, 1984).

The current model for enzyme-mediated mercury resistance is based mostly on gram-negative systems, which are better understood. In this model, Hg^{2+} is bound in the periplasmic space by the binding protein MerP. The Hg^{2+} is passed to the membrane-bound transport protein MerT, which has a higher affinity for Hg^{2+} than does MerP. MerT evidently mediates the transport of Hg^{2+} into the cytoplasm, where it is bound by mercuric reductase (Misra and Silver, 1988).

Mercuric reductase is a flavin-containing disulfide oxidoreductase which catalyzes the reduction of Hg^{2+} to metallic mercury (Hg^0) with the concomitant oxidation of NADPH, (Fox and Walsh, 1982) or NADH in MAIS (Meissner and Falkinham, 1986). Hg^0 is the volatile form of mercury, and readily diffuses from the cell and from the microbial environment. Mercuric reductase activity can be assayed by suspending mercury resistant cells in medium containing $^{203}\text{HgCl}_2$. The reductive volatilization of ^{203}Hg can be measured as the loss of radioactivity from the medium (Weiss, et al., 1977).

Enzyme-mediated mercury resistance is encoded by mer operons which are commonly carried on plasmids and transposons in both gram-negative and gram-positive bacteria. A general map for mer operons is merRopTPAD(B), where merR encodes the regulatory protein, op denotes the operator/promoter region, merT encodes the transport protein, merP encodes the periplasmic binding protein, merA encodes mercuric reductase, merD encodes a poorly characterized protein with an apparent regulatory function, and merB, present in about 10% of mer operons, encodes organomercurial lyase (Summers, 1986). Chromsomally encoded mer operons have been reported in Staphylococcus aureus (Weiss, et al., 1977), Bacillus sp. (Wang, et al., 1987), Streptomyces lividans (J. Altenbuchner, personal communication), and others (Summers, 1986). In general, the degree of sequence similarity varies among mer genes from different bacteria (Silver and Misra, 1988), but the mercuric reductases of even the most evolutionarily distant strains share 40% of their amino acid residues (Wang, et al., 1989).

It was decided to attempt to clone the MAIS mercury resistance determinant because of its potential value as a selective marker in a MAIS cloning vector and in ongoing attempts to transform MAIS by electroporation.

Further benefits might include elucidation of MAIS operon organization and regulation as well as insight into the evolutionary relationships of MAIS mer and other mer operons. Gambill and Summers (1985) constructed a cloning vector which uses Hg^{R} as the selective marker for use in Pseudomonas. Mercury resistance might be an advantageous selective marker because MAIS show a high tolerance for most antibiotics, in that HgCl_2 would be more stable in plate media than many antibiotics over the two to six weeks of incubation required for colony growth.

Summary of the Experimental Approach

Plasmid-encoded mercury resistance was described by Meissner and Falkinham (1984) in MAIS strain W262. Initially plasmids from W262 and other Hg^{R} MAIS strains were probed at low stringency with the available cloned mer regions from the gram-negative transposon Tn21 (Barrineau, et al., 1984), from Staphylococcus aureus (Ladagga, et al., 1987), and later, Streptomyces lividans (J. Altenbuchner, unpublished) in an effort to locate MAIS mer genes. Also, S. aureus merB encoding organomercurial lyase, was used as a probe in a similar manner. When these steps proved fruitless, genomic digests of Hg^{R} MAIS strains, including a Hg^{R} strain with no plasmids (P. Scott Meissner, dissertation), were probed with the cloned S. lividans mer region. Several bands of various molecular weights hybridized with the S. lividans mer probe. The appropriate genomic DNA digests were cloned and the transformants were screened by hybridization with the S. lividans mer probe and by attempts to detect Hg^{R} expression. These efforts proved futile.

METHODS

Promoter Probe Experiments

To demonstrate Mycobacterium avium, M. intracellulare, and M. scrofulaceum group (MAIS) DNA contains promoters able to function in Escherichia coli, MAIS total DNA was cloned into promoter probe vector pMC1403 (Casadaban, 1980), a plasmid with a lac region from which the promoter has been deleted. Total genomic DNA was isolated from MAIS strain SC2 by a modification (John L. Johnson, personal communication) of the method of Marmur (1961), with antibiotic treatment during growth before lysis (Crawford and Bates, 1979). Partial restriction digestions with Sau3A of mycobacterial and E. coli strain DH5a (Sambrook, et al., 1989) genomic DNA control were performed as described by Sambrook, et al. (1989). The DNA was then subjected to electrophoresis on a 1% Sea Plaque low melting point agarose gel (FMC Corp., Rockland, ME). The agarose containing DNA fragments from 0.4 to 1.5 kb in size was excised and extracted with phenol (Maniatis, et al., 1982). DNA ligations and transformations were performed as described by Sambrook, et al. (1989), and the resulting white (promoter - positive) and blue (promoter - negative) transformant colonies were counted on agar plates containing Bluo-gal (BRL, Bethesda, MD) as described by Casadaban (1980).

Detection of Plasmid and Genomic mer DNA with mer Probes

Plasmid DNA and total genomic DNA of MAIS strains was probed with mer (the set of genes encoding mercury resistance) probes in an attempt to identify MAIS mer fragments. Plasmid DNA from mercury resistant MAIS

strains CL28, MD1, MD2, MD17, SC2, SC7, VA5, VA18, W39, and W262 (P. Scott Meissner, dissertation, 1984) was isolated (Meissner and Falkinham, 1986) and transferred to Zeta Probe membranes (Bio-Rad, La Jolla, CA) as described (Jucker and Falkinham, 1990). Total genomic DNA was isolated from mercury resistant MAIS strain CL29, which has no plasmids (P. Scott Meissner, dissertation) and from strain SC2 by as described above, subjected to restriction digestion with BamHI, EcoRI, HindIII, KpnI, PstI, SmaI, SphI, or XbaI, and transferred to hybridization membranes as described above. A DNA probe was made from the Pseudomonas sp. mer operon originating from Tn21 in plasmid R1 (Barrineau, et al., 1984) which had been cloned into pDG106 (Gambill and Summers, 1985). A second probe was made from the Staphylococcus aureus mer operon in pI258 which had been cloned into pPG50 (Götz, et al., 1983). Also, a probe was made from the merB region, which encodes the organomercurial lyase of the same operon. A fourth probe was made from the Streptomyces lividans mer operon which had been cloned into pJOE581.2 (Joseph Altenbuchner, unpublished).

DNA probes were prepared by removal of the cloned insert from the appropriate plasmid by restriction digestion, separation by preparative agarose electrophoresis (Jucker and Falkinham, 1990), and radiolabeling with ³⁵S-dCTP using a random primer radiolabeling kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's directions. Hybridizations were performed overnight in 4X SSPE (Sambrook, et al., 1989), 1% (w/v) SDS, 0.25% (w/v) skim milk powder, 30% formamide at 42°. With the Streptomyces probe, the parameters were as above except 20% formamide was used at 60°.

Attempts to Clone mer Using the pYW22 Expression System

A 6.5 kb BamHI or PstI restriction digest fragment from MAIS strain SC2 hybridized with the S. lividans mer probe (Table A). Attempts were made to clone these fragments.

SC2 genomic DNA was prepared as described above, digested with either BamHI or PstI, and subjected to electrophoresis on 0.8% standard (high) melting point agarose. After electrophoresis and ethidium bromide staining (Sambrook, et al., 1989) wells of about 3 cm in length were cut above and below the region around 6.5 kb containing the fragment of interest. Fresh 0.8% low melting point agarose was poured into the wells and allowed to harden. The polarity of the electrodes was reversed, and the DNA was run backwards (ie. upwards on the gel) into the low melting point agarose plugs. The DNA was excised from the gel and dialyzed in 40 volumes of water at 4°C for 10 min. Insert DNA was ligated into pUC19 (Yannisch-Perron, 1985) in low melting point agarose (Sambrook, et al., 1989). Plasmid mini-preparations (Sambrook, et al., 1989) were used to confirm that most inserts ranged near the desired 6.5 kb size.

To prepare a host strain for expression of mercury resistance, E. coli strain JM83 (Vjiera 198x) was transformed with pYW22, a plasmid bearing a gram-negative mer operon from which merA has been deleted, resulting in mercury hypersensitive cells (Wang, et al., 1987). The resulting strain, JM83 (pYW22), was then transformed with ligated DNA prepared as described above. Transformant colonies were screened both by colony hybridization (Sambrook, et al., 1989) using the S. lividans probe as described above, and by selection of Hg^R transformants on LB agar plates containing HgCl₂ as described by Wang, et al., 1987). The colony hybridization experiments were repeated using

the same probe radiolabeled with ^{32}P -dCTP. Cloned inserts from transformants that hybridized with the S. lividans mer probe by colony hybridization were analyzed further by plasmid mini-preparations, capillary transfer, and hybridization, as described above. Over 1200 recombinant colonies were screened.

RESULTS

Promoter Probe Experiments

Escherchia coli strain DH5a (Sambrook, et al., 1989) was transformed with the promoter probe pMC1403 containing inserts of mycobacterial or DH5a DNA or with uncut pMC1403. Over 500 colonies of each transformation were enumerated on Blue-gal plates. The cells transformed with the pMC1403 containing DNA from Mycobacterium avium, M. intracellulare, M. scrofulaceum group (MAIS) strain SC2 resulted in 3.9% blue transformant colonies. With DH5a DNA, 6.5% of colonies were blue, and with uncut pMC1403 DNA less than 0.1% were blue.

Detection of Plasmid-Encoded and Genomic mer DNA with mer Probes

Total plasmid DNA from nine MAIS strains and restriction endonuclease digests of genomic DNA were transferred to hybridization membranes and hybridized with radiolabeled mer DNA probes from E. coli, Staphylococcus aureus, or Streptomyces lividans. No hybridization was detected between DNA from the Hg^R MAIS and mer probes from E. coli or S. aureus. Only the genomic DNA digests which were hybridized with the S. lividans probe yielded discernible bands on the X-ray film. The strongest bands appeared as shown in Table A.

Attempts to Clone mer Using the pYW22 Expression System

Genomic DNA fragments from Hg^R MAIS strain SC2 were cloned and used to transform E. coli strain JM83(pYW22), which carries a mer operon with a deletion in merA, the gene encoding mercuric reductase. No

Table A. Apparent sizes (kb) of DNA fragments resulting from restriction endonuclease digestion of genomic DNA which hybridized with S. lividans mer.

Strain	<u>Restriction Endonuclease</u>						
	BamHI	EcoRI	KpnI	PstI	SmaI	SphI	XbaI
CL29	N/A *	9.0	N/A	7.8	1.2	N/A	N/A
SC2	6.5	14.6	7.2	6.6	12.7	12.8	8.3

* N/A - No band was observed.

recombinant transformant colonies were obtained that clearly showed mercury resistance on HgCl_2 plates. About 16 transformants were obtained which showed a signal in colony hybridization. These transformants were analyzed further by plasmid isolation, isolation of the inserts by restriction digestion, electrophoresis, capillary transfers, and hybridization with the S. lividans probe. None of the inserts showed a stronger signal than the controls on the X-ray film. Further, all the inserts that had hybridized with the S. lividans mer probe in colony hybridization were larger than 12 kb.

DISCUSSION

Promoter Probe Experiments

Mycobacterium avium, M. intracellulare, M. scrofulaceum group (MAIS) DNA cloned into the promoter probe vector pMC1403 resulted in 3.9% blue transformant colonies, as compared to 6.5% blue transformant colonies with positive control DNA from Escherichia coli strain DH5a. In pMC1403 the upstream end of the β -galactosidase gene has been deleted, including the first eight codons. Thus this non-quantitative assay result indicates that MAIS DNA can provide promoter and ribosome binding site function in E. coli, albeit at a lower efficiency than with E. coli DNA. Based on this result it was decided that there was a good probability that cloned MAIS DNA might be transcribed from the MAIS promoters in E. coli. It might have been useful to include highly unrelated DNA, such as salmon sperm DNA, as another negative control to provide an estimate of the amount of nonspecific promoter function supplied.

Detection of Plasmid-Encoded and Genomic mer DNA with mer Probes

Mercury resistance operon (mer) DNA from either E. coli or Staphylococcus aureus failed to hybridize with plasmids or restriction endonuclease digests of genomic DNA from mercury resistant MAIS strains. This is not surprising in light of the great diversity, both serologically and in DNA sequence, that has been described among mer operons of various genera (Silver and Misra, 1988). The Streptomyces lividans mer probe hybridized with several fragments from MAIS genomic restriction digests (Table A). These results are not unexpected because the streptomycetes are gram positive

bacteria with a high G+C% ratio (around 70%, Hopwood, et al., 1987) like the mycobacteria, and are considered evolutionarily close to the mycobacteria (Bradley, 1972).

Only cloned MAIS DNA inserts larger than 12 kb showed a signal in colony hybridization with the S. lividans probe, although hybridization of Southern blots of genomic DNA digests had shown bands of 6.5 kb. Higher hybridization stringencies were tried to alleviate this problem, but such attempts also resulted in failure to detect the 6.5 kb fragments. It is possible that a fragment bearing MAIS mer was cloned but never detected. A DNA probe with more sequence similarity to the MAIS mer would increase the signal - to - background ratio and should increase the chance of success.

Attempts to Clone mer Using the pYW22 Expression System

The failure to isolate any Hg^R transformants with the pYW22 system could be due to several reasons. Possibly, a DNA fragment bearing MAIS mer was never cloned. Alternately, there were problems with transcription or translation, or failure of the MAIS mercuric reductase to attain the proper conformation or to function properly in the foreign host cell. Another cloning host, such as the rapid-growing mycobacterium M. smegmatis, (Newrefccod, 1991) might be preferable.

Possible Future Approaches

As more cloned mer regions become available, especially from gram positive, high G+C % organisms, they may be useful as probes. But more immediately feasible would be to use as probes small subcloned regions of Streptomyces mer corresponding to the highly conserved active site or other

highly conserved and unique regions of mercuric reductase (Wang, et al., 1989). These smaller probes should have more sequence similarity to the MAIS mer than a probe made from the entire operon, and should exhibit a higher signal - to - noise ratio.

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