ISOLATION AND CHARACTERIZATION OF PLASMIDS FROM HUMAN AND ENVIRONMENTAL ISOLATES OF MYCOBACTERIA

by

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Biology

(ABSTRACT)

Human clinical (n=131) and environmental (n=226) isolates of the Mycobacterium avium-intracellulare and M. scrofulaceum (MAIS) complex were screened for plasmids in an effort to increase knowledge about the genetics and epidemiology of these pathogenic bacteria. Approximately 50% of the clinical MAIS isolates from New York, Maryland, Virginia, South Carolina, and Georgia contained one or more plasmids. On the basis of plasmid content, aerosol MAIS isolates more closely resembled human MAIS isolates than did MAIS isolates from the other environmental sources examined (dust, soil, sediment, and water). Plasmid profiles were remarkably heterogenous, and isolates with identical profiles were rarely encountered. However, a 115 megadalton (Md) plasmid was detected in 15 mercury resistant human and environmental isolates. In one of these isolates (M. scrofulaceum W262) the presence of the 115 Md plasmid was shown to correlate with the presence of an NAD(P)H dependent mercuric reductase. Plasmids with molecular weights of 8.8, 11.2, 14.2, 16.9, 17.9, and 18.3 Md were also common among both human and environmental isolates. On the basis of molecular weight, 36 distinct plasmids were detected; their sizes

ranged from 7 to 230 Md. It was concluded that human and environmental MAIS isolates share a number of plasmids with identical molecular weights and that plasmids can serve as useful entities in genetic and epidemiologic studies of this group of extremely slow-growing, poorly understood human and animal pathogens.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

The genus Mycobacterium consists of a heterogenous group of pathogenic and non-pathogenic, slow and rapid-growing species. Salient features of the bacteria in this genus include their acid-fast staining reaction and the high mole-percent guanosine-cytosine in their genome (Barksdale and Kim, 1977). M. leprae, the etiologic agent of some forms of leprosy, and M. tuberculosis, a human pulmonary pathogen, are undoubtedly the most renowned species of mycobacteria. However, because of declining morbidity and mortality associated with M. tuberculosis, attention is increasingly being focused on other pathogenic mycobacterial species as agents of pulmonary infections (Good, 1979). In the United States, 65% of patients with tuberculosis have disease caused by M. tuberculosis while 25% have disease caused by bacteria belonging to the M. avium-intracellulare-scrofulaceum (MAIS) complex (Good, 1980; Good and Snider, 1982). Unlike M. tuberculosis which is spread by person to person contact and whose sole reservoir of infection is man, MAIS bacteria are not transmitted from person to person and are ubiquitous inhabitants of soils and waters worldwide (Wolinsky, 1979). In the United States, the increased frequency of recovery of representatives of MAIS from water samples collected in the southeastern United States (Falkinham et al., 1980) correlates with the high frequency of persons showing evidence of prior infection (Edwards et al., 1969) and the high frequency of recovery of mycobacteria of this group from specimens submitted to state tuberculosis laboratories (Good, 1980; Good and Snider, 1982). This observation, and the lack of evidence for other modes of disease transmission (Wolinsky, 1979), suggests that environmental MAIS are a

source of human disease. However, until this hypothesis is substantiated, appropriate public health measures cannot be initiated.

If the hypothesis that MAIS-associated diseases result from contact with environmental sources is correct, then human and environmental MAIS isolates from the same geographical area should be identical. One of the long-range goals of research in this laboratory is to test this hypothesis by comparing, phenotypically and genotypically, human and environmental MAIS isolates obtained from a variety of locations along the eastern seaboard of the United States (Falkinham et al., 1980; Wendt et al., 1980; Brooks, in press).

In other human diseases which result from contact with environmental bacterial pathogens, phenotypic markers such as serotype and phagetype have served as useful tools in epidemiologic analyses . Although serotyping is a useful marker in epidemiologic studies of MAIS-associated diseases (Codias and Reinhardt, 1979; Tsang et al., 1983), this method suffers from certain limitations (Crawford et al., 1981c). For example, a significant portion of of the MAIS isolates submitted for serotyping are either untypable or autoagglutinate (Brennan et al., 1982). Similarly, the utility of phage typing (Crawford et al., 1981c) appears limited because many human MAIS isolates are resistant to the phages used in current typing schemes (Dr. Jack Crawford; personal communication).

OBJECTIVES

Aware of the inadequacy of existing MAIS epidemiological markers, we embarked on a study to screen for plasmids among human and environmental MAIS isolates. This study was initiated with several objectives in mind. These are listed below.

1) Develop methods to determine whether human or environmental MAIS isolates contain plasmids.

2) Determine the feasiblility of using MAIS plasmid profiles as epidemiological markers.

3) Determine if and how plasmids might be involved in phenotypic expression of traits such as heavy-metal resistance.

RATIONALE OF THE EXPERIMENTAL APPROACH

The following discussion considers the rationale for each of the objectives.

1) When this study began in 1980, virtually nothing was known about the extrachromosomal genetics of MAIS. Plasmids had been isolated from a few MAIS strains (Crawford and Bates, 1979), but a systematic determination of the frequency of plasmids among large numbers of human and environmental isolates from different geographical locations had not been made. Crawford had developed a cycloserine-ampicillin treatment which enabled MAIS bacteria to be gently lysed by detergents (Crawford et al., 1981a), and in 1981 a simple procedure for rapidly detecting plasmids was reported by Kado and Liu (1981). Taken together, these developments enabled us to screen for plasmids among large numbers of MAIS isolates. This study was needed because, before any further plasmid analyses could be planned, we needed to obtain basic information such as frequencies and sizes of plasmids among human and environmental MAIS isolates.

2) As mentioned earlier, lack of adequate markers has hindered epidemiological studies of MAIS-associated diseases. Plasmid profiles have served as useful markers in epidemiological studies of other bacterial genera (Farrar, 1983; Orskov and Orskov, 1983), and we wondered whether the same might hold true for studies with MAIS. Accordingly, we obtained human isolates from New York, Maryland, Virginia, South Carolina, and Georgia. We compared the plasmid profiles of these isolates with profiles of isolates from the same areas representing the following environmental sources: chicken litter, dust,

soil, sediment, water, and aerosols. If environmental MAIS cause human disease, then human and environmental isolates from the same area should have similar plasmid profiles. Further, by comparing human MAIS profiles with profiles from isolates obtained from different environmental sources, it should be possible to determine which sources makes the most significant contribution to human colonization and disease.

3) Because MAIS have been recovered from waters known to be contaminated with heavy metals (the Delaware and Chesapeake Bays, for example), it should be possible to obtain isolates resistant to mercury, cadmium, and other heavy metals. Accordingly, large numbers of human and environmental MAIS were screened for resistance to these compounds. Although many MAIS were sensitive, mercury and/or cadmium resistant isolates were identified (Falkinham et al., 1984). We had wished to obtain isolates with these phenotypes because they would be useful in studies of the physiological ecology and epidemiology of MAIS organisms. For example, in other bacterial genera the genes encoding mercury and cadmium resistance have generally been shown to be located on plasmids (Robinson and Tuovinen, 1984). We therefore believed that mercury and cadmium resistant MAIS isolates would contain plasmids that encoded these functions. The identification and characterization of such plasmids interested us for the following reasons:

a.) In studies of physiological ecology, the identification of plasmids encoding mercury and cadmium resistance would increase understanding of some of the adaptations of MAIS to polluted environments.

b.) In epidemiological studies, the identification of these plasmids would allow for more specific comparisons of extrachromosomal DNA in human and

environmental MAIS isolates. That is, instead of simply determining whether these isolates share plasmids of similar molecular weight, we would also be able to ascertain whether they shared plasmids encoding identical phenotypes.

c.) At the time of this study, no selectable plasmid-encoded phenotypes had yet been reported in MAIS (Crawford et al., 1981b; Mizuguchi et al., 1981). The identification of such markers would be essential to future studies of mycobacterial gene transmission mechanisms.

d.) Although the physiology (Summers and Silver, 1978) and genetics (Summers et al., 1978) of mercury resistance have been well characterized in a large number of bacterial genera (Foster, 1983), the mycobacteria have not been studied. Almost without exception the mechanism of resistance in other genera involves the synthesis of a mercury-volatilizing mercuric reductase (Robinson and Touvinen, 1984). The reductase gene is nearly always plasmid-encoded and in many cases the gene is carried on transposable elements (Tanaka et al., 1983). Aware of these facts, we wished to determine whether mercury resistant MAIS isolates volatilized mercury and, if so, the characteristics of their mercuric reductase. If plasmids were detected in mercury resistant isolates, we hoped to unequivocally determine which plasmids encoded the resistance. This would be done by comparing the plasmid profiles of mercury resistant isolates with those of their sensitive segregants. Overall, these studies should allow us to ascertain if and how plasmids are involved in the mercury resistance of MAIS isolates.

CHAPTER 2

PLASMID ISOLATION

INTRODUCTION

As discussed in Chapter 1, the first goal of this project was to attempt to isolate plasmids from human and environmental MAIS isolates. Since many isolates were to be tested, a simple, rapid, and reproducible isolation procedure capable of detecting large and small plasmids would be ideal. Fortunately, just such a procedure appeared in the literature in March of 1981 (Kado and Liu, 1981). The method was a simple modification of existing alkaline denaturation methods (Hansen and Olsen, 1978; Birnboim and Doly, 1979). Like these earlier procedures, the Kado and Liu method relies on a high pH step to selectively denature chromosomal DNA (Birnboim and Doly, 1979). However, Kado and Liu realized that a simple phenol extraction could replace the awkward and time-consuming neutralization steps embodied in the earlier methods (Currier and Nester, 1976; Casse et al., 1979). Since phenol, a strong acid and denaturing agent, is employed in nearly every plasmid isolation procedure, Kado and Liu (1981) believed that direct phenol addition to alkaline lysates would accomplish neutralization and denaturation in a single step. Thus, in Kado and Liu's method, neutralization and denaturation are performed in one step whereas in earlier procedures they required at least two separate manipulations (Hansen and Olsen, 1978; Casse et al., 1979). By reducing steps, Kado and Liu (1981) increased the ease and reproducibility of isolation of large plasmids which are preferentially lost during transfer (Hansen and Olsen, 1978).

MATERIALS AND METHODS

Maintenance, growth, and drug treatment of MAIS isolates. At the start of these studies in 1980, MAIS isolates were subcultured from refrigerated slants. This was accomplished by suspending a loopful of growth into 10 ml of Middlebrook 7H9 broth (BBL Microbiological Systems, Cockeysville, Md) containing 0.4% glycerol and 10% OADC enrichment (OADC was prepared by us as described in Chapter 5). We refer to this medium as MGE broth. These primary broths were incubated at 30°C with occassional vortexing until growth was at least 10^8 cells/ml. This usually required 2-4 weeks. The primary broths were then refrigerated indefinitely. For plasmid isolations, one or more secondary 10 ml MGE broths were inoculated with 1.0 ml from primary broths. The incubation temperature for all work was 30°C. All cultures were incubated statically, with vortexing every few days until cultures reached late log phase. Cultures were then subjected to the 18 hour drug treatment described by Crawford (1981a). A 1:10 dilution of a freshly prepared solution of Ampicillin (1 mg/ml) and D-cylcoserine (10 mg/ml) was added to cultures which were then placed on a roller drum apparatus in order to maintain a homogenous solution during the 18 hour treatment. Often, by the end of the treatment, cultures that originally were uniformally turbid had become clumped.

Plasmid isolation. Lysing solution (LS; 3% sodium dodecyl sulfate in 50 mM Tris; pH adjusted to 12.5 with 3 N NaOH) and E buffer (40 mM Tris and 2 mM sodium EDTA; pH adjusted to 7.9 with glacial acetic acid) were prepared as described by Kado and Liu (1981). Phenol saturated with 0.5 M NaCl was

prepared by mixing 500 ml phenol with 50 ml 0.5 M NaCl. This solution was then maintained at ambient temperature and oxidation was prevented by adding 0.1% 8-hydroxyquinoline as described by Maniatis (1982). Drug treated cells were harvested by centrifugation (8,000 x g for 10 min). Approximately 10^{10} cells (from 10 to 30 ml of broth culture) were suspended in 0.3 ml E buffer and lysed by addition of 2 ml of LS. Lysates were incubated at 60°C for 20 min, cooled at room temperature for 5 min, and then approximately 2 volumes of phenol (saturated with 0.5 M NaCl) was added. Lysates were then placed on a roller drum apparatus (New Brunswick Scientific Co., New Brunswick, NJ) and were rotated for 5 min at 4 rpm. Lysates were then cleared by centrifugation at 10,000 x g for at least 60 min. For vertical or horizontal electrophoresis, 50 ul of the aqueous phase was mixed with 10 ul of the tracking dye used by Kado and Liu (1981) or with 10 ul of 20% Ficoll (Sigma) in 10 mM Tris containing 1 mM EDTA and 0.07% bromophenol blue (pH 8.0).

Restriction endonuclease analysis. To prepare sufficient plasmid DNA for endonuclease restriction analysis I simply increased the volumes of the standard mycobacterial isolation method (described above) by a factor of 10. Further, the phenol-extracted lysates were cleared by centrifugation at 10,000 x g for at least 2 hours. The aqueous phase was then removed and 1/10 volume of 3 M sodium acetate was added, followed by 2 volumes of cold (4°C) ethanol. The precipitated plasmid DNA was then pelleted by centrifugation at 10,000 x g for 30 min. The supernatant was discarded and the pellet was washed with cold 80% ethanol for 5 min. The pellet was then air dried for 10 min and suspended in 1 ml of 10 mM Tris containing 1 mM EDTA (pH 8.0). Restriction was then performed. All restriction enzymes were from Bethesda Research Labs (BRL;

Gaithersburg, MD) and they were used in accordance with the instructions provided by BRL.

Agarose gel electrophoresis. All electrophoresis was carried out with either a vertical slab apparatus (SE 600; Hoefer Instruments) or with a horizontal apparatus (model HO; BRL). Vertical gels were prepared by dissolving DNA grade agarose (BioRad Labs, Richmond, CA) in 89 mM Tris, 89 mM boric acid buffer (pH 8.2) containing 2.5 mM EDTA (TBE). The running buffer was also TBE. Slab dimensions were 16 cm by 18 cm by 3 mm. Gels were run with an agarose concentration of 0.7% at a constant voltage of 10 V/cm. Electrophoresis was terminated when the tracking dye had reached the bottom of the gel. Horizontal gels were prepared by dissolving agarose in 270 ml distilled water. Just prior to pouring gels, 30 ml of a 10X stock solution of E buffer was added. Gel dimensions were 20 cm by 25 cm by 4 mm. Gels were run submersed in E buffer at a constant voltage of 4 V/cm for 12-18 hours. Vertical and horizontal gels were stained for 10 min in a 0.5 ug/ml solution of ethidium bromide. After rinsing under tap water for 2 minutes, plasmids were visualized with a Fotodyne transilluminator and were photographed with a Polaroid MP-4 camera and type 667 Polaroid film.

Table 1. List of human and environmental MAIS isolates.

ISOLATE[a]		PBMA[b]	TYPE[c]	SOURCE[d]	STATE[e]
MD	1	1	H	MONTGOMERY	MD
MD	2	1	H	BALTIMORE CITY	MD
MD	3	1	H	BALTIMORE CITY	MD
MD	4	1	H	BALTIMORE CO.	MD
MD	5	0	H	BALTIMORE CITY	MD
MD	6	1	H	BALTIMORE CITY	MD
MD	7	1	H	TALBOT	MD
MD	8	1	H	SUSSEY	DE
MD	9	0	H	WICOMICO	MD
MD	10	1	H	BALTIMORE CO.	MD
MD	11	1	H	PRINCE GEORGE'S	MD
MD	12	1	H	BALTIMORE CO.	MD
MD	13	1	H	BALTIMORE CO.	MD
MD	14	0	H	BALTIMORE CO.	MD
MD	15	0	H	BALTIMORE CO.	MD
MD	16	1	H	BALTIMORE CITY	MD
MD	17	1	H	BALTIMORE CITY	MD
MD	18	0	H	BALTIMORE CITY	MD
MD	19	0	H	MONTGOMERY	MD
MD	20	0	H	BALTIMORE CO.	MD
MD	21	1	H	BALTIMORE CITY	MD
MD	22	1	H	VINELAND	NJ
MD	23	0	H	BALTIMORE CITY	MD
MD	24	1	H	BALTIMORE CITY	MD
MD	25	1	H	DORCHESTER	MD
MD	28	1	H	EISSELE[*]	MD

[*]Human isolate Eissele provided by D. Glasser.

NY	15	1	H	GRUFT	NY
NY	76	1	H	GRUFT	NY
NY	157	0	H	GRUFT	NY
NY	788	0	H	GRUFT	NY
NY	1354	0	H	GRUFT	NY
NY	1599	1	H	GRUFT	NY
NY	1831	0	H	GRUFT	NY
NY	1832	0	H	GRUFT	NY
NY	2132	1	H	GRUFT	NY
NY	2672	0	H	GRUFT	NY
NY	2731	1	H	GRUFT	NY
NY	3213	1	H	GRUFT	NY
NY	3725	1	H	GRUFT	NY
NY	5167	1	H	GRUFT	NY
NY	6373	0	H	GRUFT	NY
NY	6644	1	H	GRUFT	NY
NY	6645	1	H	GRUFT	NY
NY	7897	0	H	GRUFT	NY
NY	8032	0	H	GRUFT	NY
NY	8628	1	H	GRUFT	NY
NY	9021	1	H	GRUFT	NY
NY	9319	1	H	GRUFT	NY
NY	58125	0	H	GRUFT	NY

Table 1. continued

ISOLATE		PDNA	TYPE	SOURCE	STATE
VA	1	0		RICHMOND	VA
VA	2	1	H	RICHMOND	VA
VA	3	1	H	RICHMOND	VA
VA	4	0	H	RICHMOND	VA
VA	5	1	H	RICHMOND	VA
VA	6	1	H	RICHMOND	VA
VA	7	0	H	PETERSBURG	VA
VA	8	0	H	PETERSBURG	VA
VA	9	1	H	HAMPTON	VA
VA	10	1	H	HAMPTON	VA
VA	11	0	H	HAMPTON	VA
VA	12	1	H	HAMPTON	VA
VA	13	0	H	HAMPTON	VA
VA	14	1	H	NORFOLK	VA
VA	15	1	H	NORFOLK	VA
VA	16	0	H	NORFOLK	VA
VA	17	0	H	PORTSMOUTH	VA
VA	18	1	H	VIRGINIA BEACH	VA
VA	19	0	H	COLONIAL HEIGHT	VA
VA	20	0	H	COLONIAL HEIGHT	VA
VA	21	1	H	ACCOMA	VA
VA	22	1	H	HEYES	VA
VA	230	0	H	N. WARREN	VA
VA	240	1	H	N. WARREN	VA
VA	250	1	H	N. WARREN	VA
VA	270	1	H	N. WARREN	VA
VA	280	1	H	N. WARREN	VA
VA	290	1	H	N. WARREN	VA
VA	300	0	H	N. WARREN	VA
VA	310	1	H	N. WARREN	VA
VA	320	0	H	N. WARREN	VA
VA	330	1	H	N. WARREN	VA
VA	340	0	H	N. WARREN	VA
VA	350	0	H	N. WARREN	VA
VA	360	0	H	N. WARREN	VA
VA	370	0	H	N. WARREN	VA
VA	380	1	H	N. WARREN	VA
VA	390	1	H	N. WARREN	VA
VA	400	1	H	N. WARREN	VA
VA	410	1	H	N. WARREN	VA
VA	420	1	H	N. WARREN	VA
VA	430	1	H	N. WARREN	VA
VA	440	0	H	N. WARREN	VA
VA	450	0	H	N. WARREN	VA
VA	470	1	H	N. WARREN	VA
VA	480	0	H	N. WARREN	VA
VA	490	1	H	N. WARREN	VA
VA	500	0	H	N. WARREN	VA
VA	510	0	H	N. WARREN	VA

Table 1. continued

ISOLATE		PDNA	TYPE	SOURCE	STATE
GA	1	1	H	FLOYD	GA
GA	2	1	H	RICHMOND	GA
GA	3	1	H	RICHMOND	GA
GA	4	0	H	UNKNOWN	GA
GA	5	0	H	RICHMOND	GA
GA	6	0	H	CHATHAM	GA
GA	7	0	H	CHATHAM	GA
GA	8	1	H	CHATHAM	GA
GA	9	1	H	RICHMOND	GA
GA	10	0	H	COLUMBIA	GA
GA	11	1	H	CHATHAM	GA
GA	13	1	H	CHATHAM	GA
GA	14	0	H	BULLOCH	GA
GA	15	0	H	RICHMOND	GA
GA	17	1	H	RICHMOND	GA
GA	18	0	H	RICHMOND	GA
GA	22	0	H	BULLOCH	GA
GA	24	0	H	UNKNOWN	GA
GA	25	1	H	CHATHAM	GA
GA	26	1	H	UNKNOWN	GA
GA	27	0	H	RICHMOND	GA
GA	28	0	H	RICHMOND	GA
GA	29	1	H	EFFINGHAM	GA
GA	30	0	H	RICHMOND	GA
GA	31	1	H	RICHMOND	GA
SC	1	0	H	ANDERSON	SC
SC	2	1	H	ANDERSON	SC
SC	3	1	H	ANDERSON	SC
SC	4	0	H	ANDERSON	SC
SC	5	0	H	ANDERSON	SC
SC	6	0	H	ANDERSON	SC
SC	7	1	H	ANDERSON	SC
SC	8	1	H	OCONEE	SC
SC	9	0	H	EDGEFIELD	SC
LR	25	1	H	CRAWFORD	
LR	113	1	H	CRAWFORD	
LR	145	1	H	CRAWFORD	
LR	154	1	H	CRAWFORD	
LR	196	1	H	C:TMC 1312	
LR	199	1	H	C:TMC 1321	
LR	200	1	H	C:TMC 1323	

Table 1. continued

ISOLATE		PDNA	TYPE	SOURCE	STATE
JR	74	0	D	RICHMOND	VA
JR	77	1	D	RICHMOND	VA
JR	120	0	W	RICHMOND	VA
JR	129	0	W	RICHMOND	VA
JR	138	0	W	RICHMOND	VA
JR	177	0	D	RICHMOND	VA
JR	223	0	W	RICHMOND	VA
JR	266	0	D	RICHMOND	VA
JR	318	0	W	RICHMOND	VA
JR	326	0	W	RICHMOND	VA
JR	401	0	W	RICHMOND	VA
JR	463	0	D	RICHMOND	VA
JR	522	0	W	RICHMOND	VA
JR	584	0	W	RICHMOND	VA
JR	686	0	W	RICHMOND	VA
JR	820	0	W	RICHMOND	VA
JR	959	0	W	RICHMOND	VA
JR	1088	0	W	RICHMOND	VA
JR	1360	0	D	RICHMOND	VA
JR	1361	0	D	RICHMOND	VA
JR	1369	0	D	RICHMOND	VA
JR	1385	0	D	RICHMOND	VA
JR	1388	0	D	RICHMOND	VA
JR	1394	0	W	RICHMOND	VA
JR	1470	0	D	RICHMOND	VA
JR	1472	0	D	RICHMOND	VA
JR	1505	0	D	RICHMOND	VA
JR	1537	0	D	RICHMOND	VA
JRS	3	0	S	RICHMOND	VA
JRS	16	0	S	RICHMOND	VA
JRS	17	0	S	RICHMOND	VA
JRS	18	0	S	RICHMOND	VA
JRS	19	0	S	RICHMOND	VA
JRS	20	0	S	RICHMOND	VA
RM	13	0	M	RICHMOND	VA
RM	16	0	M	RICHMOND	VA
RM	20	0	M	RICHMOND	VA
RM	50	0	M	RICHMOND	VA
RM	66	0	M	RICHMOND	VA
RM	69	0	M	RICHMOND	VA
RM	71	0	M	RICHMOND	VA
RM	72	0	M	RICHMOND	VA
RM	74	0	M	RICHMOND	VA
RM	87	0	M	RICHMOND	VA
RM	90	0	M	RICHMOND	VA
RM	97	0	M	RICHMOND	VA
RM	107	0	M	RICHMOND	VA
RM	109	0	M	RICHMOND	VA
RM	115	0	M	RICHMOND	VA
RM	116	0	M	RICHMOND	VA

Table 1. continued

ISOLATE		PDNA	TYPE	SOURCE	STATE
W	33	0	W	NEWPORT NEWS	VA
W	39	1	W	HOPEWELL	VA
W	136	0	W	PASS CHRISTIAN	MI
W	173	0	W	SAMPIT RIVER	NY
W	188	0	W	DISMAL SWAMP	VA
W	200	0	W	CH. MENTOUR BR.	LA
W	202	0	W	BELL ISLE	LA
W	208	0	W	ST. LOUIS BRIDG	MI
W	210	0	W	PASS CHRISTIAN	MI
W	213	0	W	POINT CLEAR	AL
W	216	0	W	FORT PIKE	LA
W	219	0	W	DAUPHIN ISLE	AL
W	220	0	W	MOBILE BAY	AL
W	225	0	W	WAVELAND BEACH	FL
W	239	1	W	MIAMI BEACH	FL
W	240	1	W	MIAMI BEACH	FL
W	243	1	W	MIAMI BEACH	FL
W	244	0	W	ROANOKE ISLAND	NC
W	245	1	W	WEST ROANOKE IS	NC
W	250	0	W	NORTH ANDOVER	MA
W	251	0	W	POWELL'S CR.	VA
W	254	0	W	BALTIMORE HARB.	MD
W	256	0	W	DELAWARE BAY	MD
W	257	1	W	DELAWARE BAY	MD
W	258	1	W	DELAWARE BAY	MD
W	259	1	W	DELAWARE BAY	MD
W	260	0	W	CHESTER R.	VA
W	261	1	W	CORSICE R.	VA
W	262	1	W	LONGFORD CR.	VA
W	266	0	W	CLAREMONT BEACH	VA
W	267	0	W	CLAREMONT BEACH	VA
W	269	0	W	BALTIMORE HARB.	MD
W	270	0	W	DELAWARE BAY	MD
W	271	1	W	DELAWARE R.	MD
W	274	0	W	DELAWARE BAY	MD
W	275	0	W	CHESTER R.	VA
W	276	0	W	NORTH ANDOVER	MA
W	277	0	W	CLAREMONT BEACH	VA
W	278	0	W	DELAWARE BAY	MD
W	279	1	W	DELAWARE BAY	MD
W	280	0	W	NEWPORT NEWS	VA
W	284	0	W	CLAREMONT BEACH	VA
W	285	0	W	POWELL'S CR.	VA
W	288	0	W	POTOMAC R.	MD
W	289	0	W	POTOMAC R.	MD
W	290	1	W	POTOMAC R.	MD
W	291	1	W	POTOMAC R.	VA
W	294	0	W	NEPTUNE BEACH	FL
W	295	0	W	OREGON INLET	NC
W	340	0	W	NEWPORT NEWS	VA
W	354	0	W	LAVONIA	GA
W	607	0	W	CANTON	NJ

Table 1. continued

ISOLATE		PDNA	TYPE	SOURCE	STATE
W	617	0	W	MAURICETOWN	
W	638	0	W	POTOMAC R.	MD
W	1032	0	W	POCOMOKE R.	VA
W	1033	1	W	ASHBY	MA
W	1034	0	W	DIXIE(SAWN'E R)	FL
W	1035	0	W	FRANKLIN CO.	FL
W	1039	0	W	FRANKLIN CO.	MA
W	1049	0	W	GULF CO.	FL
W	1050	0	W	OYSTER	VA
W	1051	0	W	TAYLOR	FL
W	1053	0	W	TAYLOR	FL
DE	6	0	W	DELAWARE R.	DE
DE	10	1	W	DELAWARE R.	DE
DE	11	1	W	DELAWARE R.	DE
DE	14	0	W	DELAWARE R.	DE
DE	17	1	W	DELAWARE R.	DE
DE	20	1	W	DELAWARE R.	DE
DE	25	0	W	DELAWARE R.	DE
S	1	1	A	RICHMOND	VA
S	2	1	A	RICHMOND	VA
S	3	1	A	RICHMOND	VA
S	4	1	A	RICHMOND	VA
S	5	1	A	RICHMOND	VA
S	6	1	A	RICHMOND	VA
S	7	1	A	RICHMOND	VA
S	8	1	A	RICHMOND	VA
S	9	0	A	RICHMOND	VA
S	10	1	A	RICHMOND	VA
S	11	1	A	RICHMOND	VA
S	12	1	A	RICHMOND	VA
S	13	0	A	RICHMOND	VA
S	14	0	A	RICHMOND	VA
S	15	0	W	RICHMOND	VA
S	16	0	W	RICHMOND	VA
S	17	1	W	RICHMOND	VA
S	18	1	W	RICHMOND	VA
S	19	0	W	RICHMOND	VA
S	20	0	A	RICHMOND	VA
S	21	1	A	RICHMOND	VA

Table 1. continued

ISOLATE		PBNA	TYPE	SOURCE	STATE
SR	33	0	S	CHATHAM	GA
SR	50	0	S	EFFINGHAM	GA
SR	101	0	S	SCREVEN	GA
SR	129	0	S	SCREVEN	GA
SR	131	0	S	SCREVEN	GA
SR	137	0	S	BURKE	GA
SR	151	0	S	BURKE	GA
SR	162	0	S	BURKE	GA
SR	164	0	S	BURKE	GA
SR	165	0	S	BURKE	GA
SR	223	0	S	ELBERT	GA
SR	256	0	S	HART	GA
SR	257	0	S	HART	GA
SR	265	0	S	HART	GA
SR	266	0	S	HART	GA
SR	268	0	S	HART	GA
SR	334	0	S	STEPHENS	GA
SR	353	1	W	CHATHAM	GA
SR	424	0	S	COLUMBIA	GA
SR	425A	0	S	COLUMBIA	GA
SR	425B	0	S	COLUMBIA	GA
SR	462	0	S	CHATHAM	GA
SR	481	1	S	CHATHAM	GA
SR	493	1	S	BURKE	GA
SR	495A	0	S	BURKE	GA
SR	495B	0	S	BURKE	GA
SR	507	0	S	COLUMBIA	GA
SR	508	0	S	COLUMBIA	GA
SR	511	0	S	COLUMBIA	GA
SR	512	0	S	COLUMBIA	GA
SR	516	0	S	RABUN	GA
SR	521	0	W	CHATHAM	GA
SR	522	1	W	CHATHAM	GA
SR	524	0	W	CHATHAM	GA
SR	525	0	W	CHATHAM	GA
SR	540	0	W	EFFINGHAM	GA
SR	541	0	W	EFFINGHAM	GA
SR	546	0	S	RABUN	GA
SR	550	0	S	RABUN	GA
SR	558	0	S	STEPHENS	GA
SR	599	0	S	ANDERSON	SC
SR	607	0	S	RABUN	GA
SR	614A	0	S	RABUN	GA
SR	614B	0	S	RABUN	GA
SR	618	0	W	SCREVEN	GA
SR	622	0	W	BURKE	GA
SR	634	0	S	ANDERSON	SC
SR	660	0	W	CHATHAM	GA
SR	666	1	W	CHATHAM	GA
SR	670	0	W	EFFINGHAM	GA
SR	686	0	W	ELBERT	GA
SR	692	0	W	RABUN	GA
SR	699	0	S	CHATHAM	GA
SR	703	0	S	EFFINGHAM	GA
SR	725	0	S	HART	GA
SR	798	0	W	RICHMOND	GA
SR	811	0	W	RICHMOND	GA
SR	814	0	W	EFFINGHAM	GA
SR	823	0	S	EFFINGHAM	GA

Table 1. continued

ISOLATE		PDNA	TYPE	SOURCE	STATE
TR	65	0	S	TOMBIGBEE R.	AL
TR	76	0	S	TOMBIGBEE R.	AL
TR	80	0	S	TOMBIGBEE R.	AL
TR	133	0	S	TOMBIGBEE R.	AL
TR	156	0	S	TOMBIGBEE R.	AL
TR	157	0	S	TOMBIGBEE R.	AL
TR	198	0	S	TOMBIGBEE R.	AL
TR	200	0	S	TOMBIGBEE R.	AL
TR	214	0	S	TOMBIGBEE R.	AL
TR	266	0	S	TOMBIGBEE R.	AL
TR	289	0	S	TOMBIGBEE R.	MI
TR	365	0	S	TOMBIGBEE R.	MI
TR	381	0	S	TOMBIGBEE R.	AL
TR	452	0	S	TOMBIGBEE R.	AL
TR	500	0	W	TOMBIGBEE R.	MI
TR	502	0	W	TOMBIGBEE R.	MI
TR	615	1	W	TOMBIGBEE R.	MI
TR	663	1	S	TOMBIGBEE R.	MI
TR	677	0	S	TOMBIGBEE R.	MI
TR	880	0	S	TOMBIGBEE R.	MI
TR	889	0	S	TOMBIGBEE R.	MI
CL	14	0	C	PRINCE EDWARD	VA
CL	25	1	C	PRINCE EDWARD	VA
CL	26	1	C	PRINCE EDWARD	VA
CL	28	1	C	PRINCE EDWARD	VA
CL	29	0	C	PRINCE EDWARD	VA

Legend:

[a]"Isolate" refers to the letter and number designation of each isolate.

[b]"PDNA" indicates the plasmid content of each isolate; where 1 or 0 designate the presence or absence of plasmids, respectively.

[c]"Type" refers to the type of isolate; where H, A, C, D, M, S and W refer to human, aerosol, chicken litter, dust, sediment, soil, and water isolates, respectively.

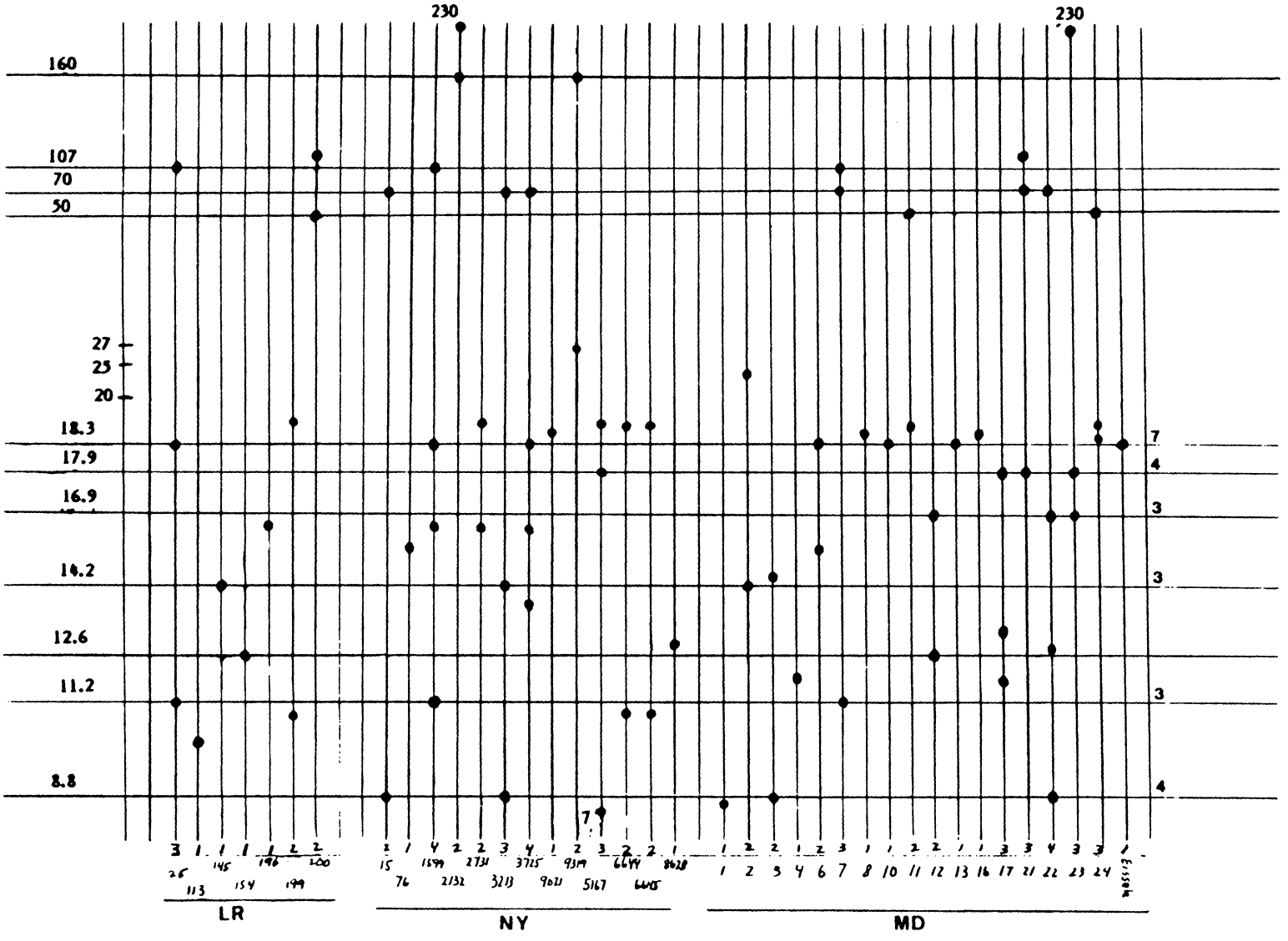
[d]"Source" refers to the region from which isolates were obtained. Generally, the county (or river) from which isolates came is given. However, in cases where this information was not available, the names of the people most closely associated with the isolates is given: N. Warren signifies that isolates were provided by Nancy Warren of Consolidated Labs, Richmond, VA; Gruft indicates that isolates were provided by Howard Gruft; Crawford indicates that Dr. Jack Crawford provided the isolates, and C:TMC signifies that Crawford provided us with isolates that he originally acquired from the Trudeau Memorial Collection.

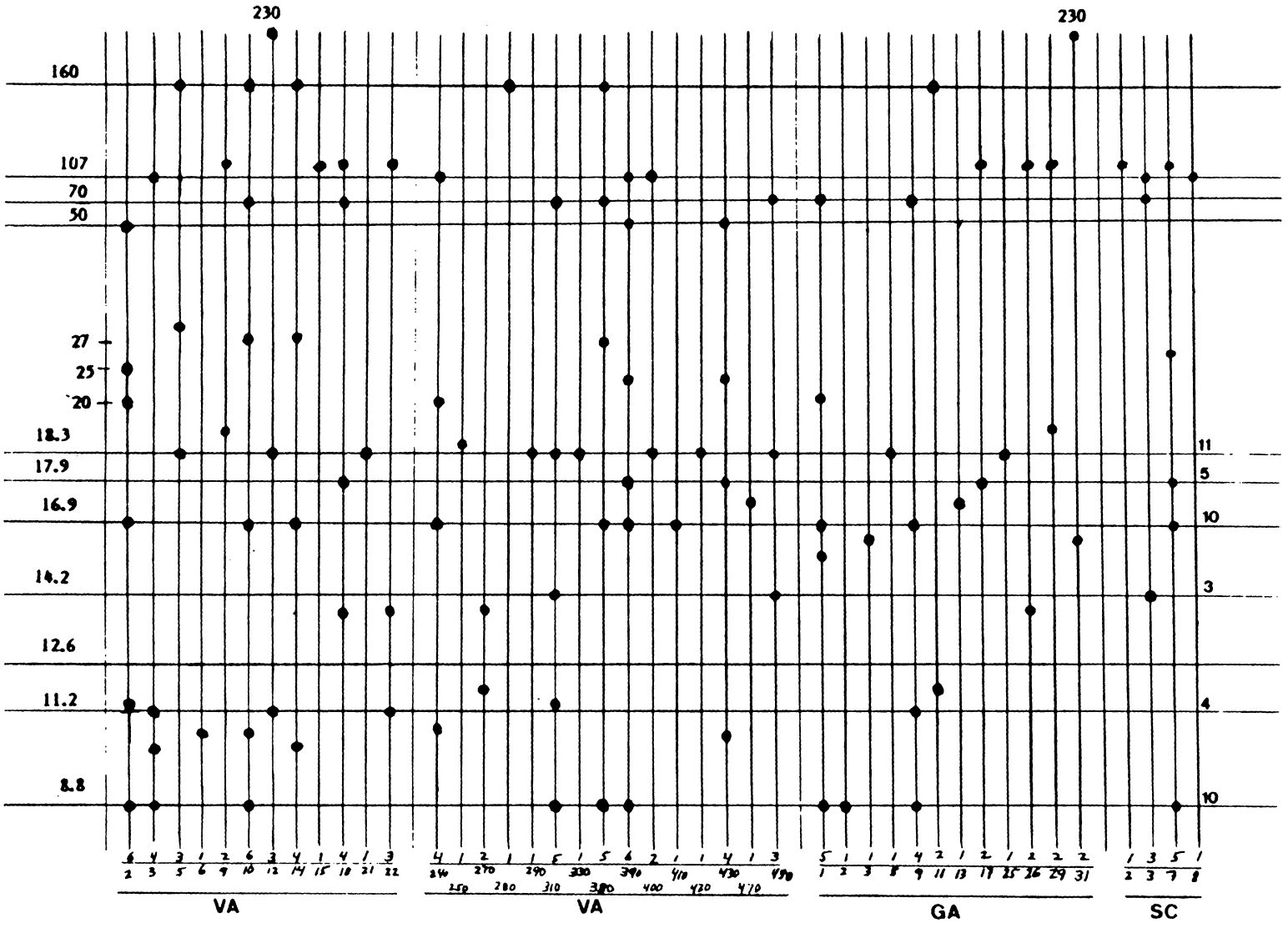
[e]"State" refers to the state from which isolates were obtained (where applicable).

Figure 1. Plasmid profiles of human and environmental MAIS isolates.

Each vertical line contains the plasmid profile of an isolate. Black dots represent plasmid bands observed during agarose gel electrophoresis. At the base of each vertical line the total number of circles (plasmids) is given, followed by the number and letter designation of each isolate.

Lines running horizontally represent the location of plasmids whose molecular weights are given (in megadaltons) at the left edge of each horizontal line. Numbers on the right side of the horizontal lines indicate how many circles (plasmids) occurred along that line. On the last page of Figure 1, the numbers in parenthesis indicate the total number of plasmids observed along each horizontal line.


230
230
160
107
70
50
27
25
20
18.3
17.9
16.9
14.2
12.6
11.2
8.8
7
4
3
3
3
4
LR
NY
MD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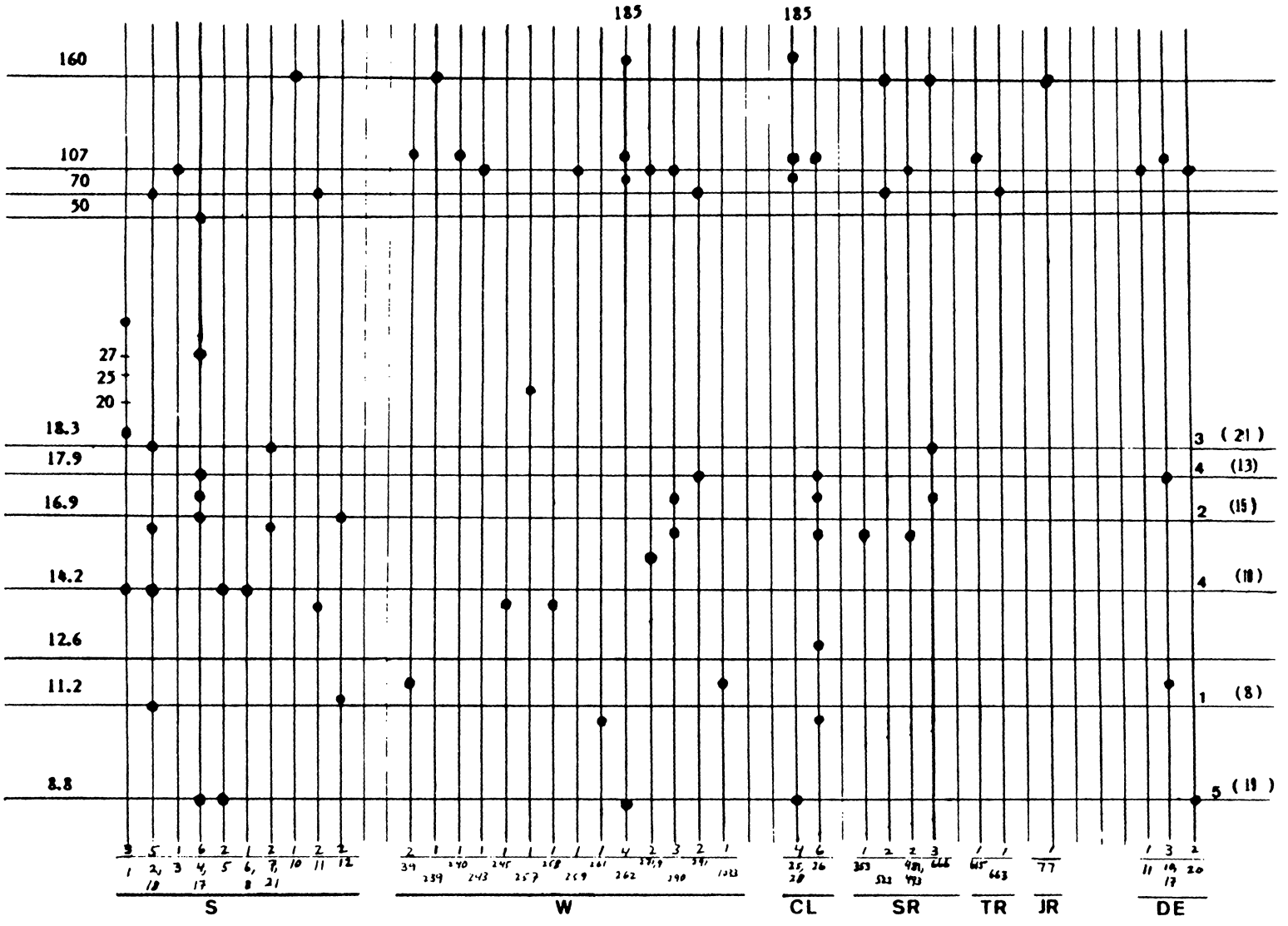
VA
VA
GA
SC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18.3
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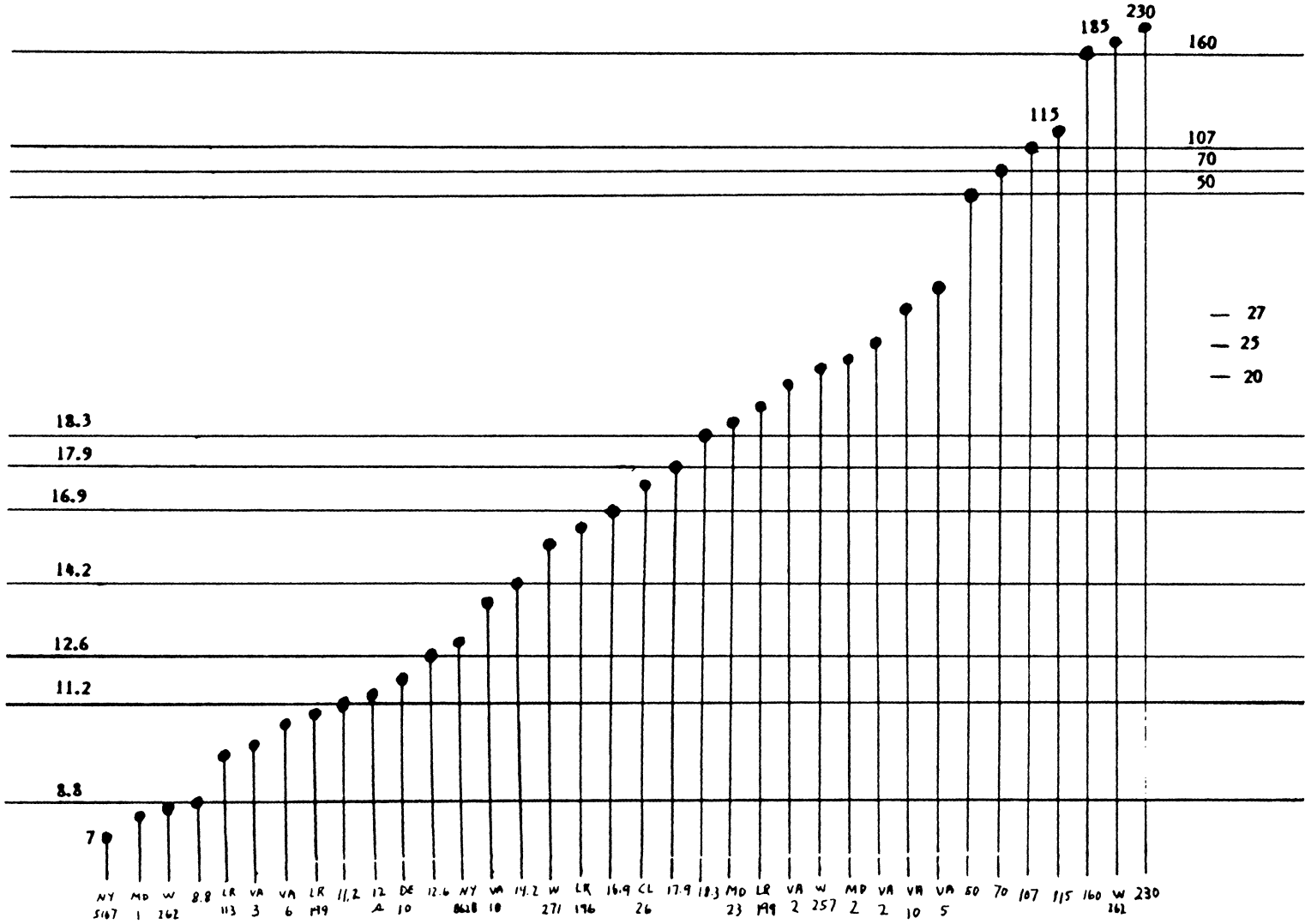
16.9
14.2
12.6
11.2
8.8
3 (21)
4 (13)
2 (15)
4 (18)
1 (8)
5 (19)
S
W
CL
SR
TR
JR
DE

Figure 2. Uniquely-sized plasmids detected in human and environmental MAIS isolates.

The top of each vertical line contains a black dot which represents a plasmid band observed following agarose gel electrophoresis. At the bottom of each vertical line the letter and number designation of the isolate corresponding to each plasmid band is given. In many cases (e.g. the 8.8, 11.2, 12.6, and 14.2 Md plasmids) the size of the plasmid is given at the base of the vertical line instead of the isolate designation. This is because so many isolates contained that particular plasmid that it would not be feasible to list each isolate (21 isolates contained an 18.3 Md plasmid for example).

Lines running horizontally represent the location of plasmids whose molecular weights are given (in megadaltons) at the left or right edge of each line. In some cases (i.e. the 7, 115, 185, and 230 Md plasmids) the size of the plasmid is given next to the black dot.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12.6
11.2
8.8
7
NY 5167
MD 1
W 162
8.8
LR 113
VA 3
VA 6
LR M9
11.2
12 A
DE 10
12.6
NY 8628
VA 18
14.2
W 271
LR 196
16.9
CL 26
17.9
18.3
MD 23
LR 199
VA 2
W 257
MD 2
VA 2
VA 10
VA 5
50
70
107
115
160
W 162
230

RESULTS AND DISCUSSION

Despite the apparent simplicity of the Kado and Liu (1981) method, I repeatedly failed to detect plasmids in plasmid-positive mycobacterial control strains. As it turned out, the main reason for this lack of success was a misleading statement made by Kado and Liu (1981) on page 1366 of their report, "(after adding phenol) the solution was emulsified by shaking briefly". While I recognized that "shaking" was detrimental because of shear forces (Hansen and Olsen, 1978), I also found that "emulsification" was undesirable. In isolation attempts that had failed, I had manually emulsified extracts. To reduce the need for tedious hand emulsifications, I decided to try to rotate extracts in tubes using a mechanical roller-drum apparatus. Using this device, the Kado and Liu (1981) method worked exactly as they had claimed. Apparently, after the phenol is added to the alkaline lysate, the resulting biphasic mixture must be treated very gently to avoid extracting plasmids out of the aqueous phase. Kado and Liu's (1981) misleading statement belies the caution actually needed at the phenol-extraction step in their simple and rapid procedure. Additionally, it is worth noting that mechanical rotation devices are becoming increasingly common in labs performing plasmid isolations.

Once I had successfully used the Kado and Liu (1981) method to isolate large and small plasmids from plasmid-positive mycobacterial control strains, I was ready to test mycobacterial isolates for the presence of plasmids. However, in order to test a given bacterial isolate for plasmids, one must first be able to lyse the cells gently with a detergent such as sodium dodecyl sulfate (SDS). Like

most gram positive organisms, mycobacteria are completely resistant to lysis by SDS. However, mycobacteria are also resistant to lysozyme. This is probably due to the high lipid content of their cell walls which limits access of the enzyme to peptidoglycan. Fortunately, Crawford had developed a drug treatment which rendered MAIS isolates susceptible to SDS lysis (Crawford et al., 1981a). The method involved an 18 hour exposure of late log phase MAIS cultures to 1 mg/ml of D-cycloserine and 100 ug/ml Ampicillin. These agents inhibit peptidoglycan crosslinking and, after about 18 hours of exposure, the cell walls of MAIS bacteria are weakened to the point where they will rupture in the presence of SDS. However, in order for these drugs to weaken the cell wall via inhibition of crosslinking, cultures must be actively growing at the time of drug addition. Since the most rapid growth of MAIS isolates is obtained in nonselective M7H9 broth containing 0.4% glycerol and 10% OADC enrichment (MGE broth), this presently appears to be the only medium in which MAIS can be grown for plasmid isolations. Thus, for all of the isolations I performed, MAIS were grown in MGE broth.

The first MAIS isolates I worked with were the plasmid-containing LR/TMC strains provided by Dr. Jack Crawford (Table 1). During preliminary work with these strains, I developed some minor modifications (Meissner and Falkinham, 1983) of the Kado and Liu (1981) procedure. These modifications consisted of extracting lysates with phenol (saturated with 0.5 M NaCl) instead of phenol/chloroform and lowering the pH of the lysis solution (LS) from 12.6 to 12.5. I substituted phenol/NaCl for chloroform because lysates extracted with the former had much clearer backgrounds during agarose gel electrophoresis than did lysates extracted with the latter. I lowered the pH of the lysing

solution because I used a higher ratio of LS than that used by Kado and Liu. If I had used a higher LS ratio without lowering the pH, this could have resulted in the irreversible denaturation of plasmid DNA (Birnboim and Doly, 1979). I used a higher LS ratio than Kado and Liu (1981) because they suggested suspending cells from a 10 ml broth culture in 1 ml E buffer followed by 2 ml LS. However, they were working with gram negative bacteria such as Escherichia coli which are extremely SDS-sensitive and yield cleared lysates devoid of unlysed cells. When I lysed an equivalent volume of MAIS culture, I always noted less viscosity (as judged by qualitative visual observation) and I never observed the cleared lysates indicative of complete lysis of all cells. Because of these differences, I could not apply a protocol developed for gram negative bacteria directly to the gram positive mycobacteria. Therefore, instead of suspending a 10 ml MAIS culture in 1 ml of E buffer, I only used 0.3 ml. Bacteria were still lysed in 2 ml of LS, as recommended by Kado and Liu (1981). Essentially, I compensated for the reduced lysis of MAIS by reducing the net volume of the lysate. My "standard" mycobacterial isolation contained approximately 10^{10} cells which were suspended in 0.3 ml of E buffer and were lysed by the addition of 2 ml of LS. For non-MAIS bacteria, I followed Kado and Liu's (1981) "standard" protocol (10^{10} cells suspended in 1 ml E buffer and lysed with 2 ml of LS).

I screened for plasmids among human (n=131) and environmental (n=226) MAIS isolates. Table 1 contains data indicating which isolates were found to be plasmid-positive, and Figure 1 displays the plasmid profiles of these isolates. I identified 36 distinct plasmids based on their mobilities following agarose gel electrophoresis (Figure 2). The molecular weights of these plasmids ranged from

7 to 230 megadaltons (Md) (Figure 2). Like Crawford, I rarely encountered isolates containing plasmids smaller than about 9 Md (Crawford and Bates, 1981b; Crawford, personal communication). This contrasts with the work of Mizuguchi (1981) who isolated a 2 Md plasmid from a Japanese clinical isolate of M. intracellulare. Apparently, small plasmids are uncommon among human and environmental MAIS isolates from the eastern United States. My failure to detect small plasmids was not related to my plasmid isolation technique since I was able to routinely isolate these plasmids from E. coli strain V517 (Macrina et al., 1978).

Like Crawford (Crawford et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C 209, p. 306), I found that plasmids in the 10 to 20 Md size range were the most common. In fact, 5 plasmids in this range were detected in chicken litter isolate CL26 (Fig. 1). Since similar plasmids cannot be stably maintained in a single bacterial clone because of incompatability (Day, 1982), it is probable that CL26 harbors 5 distinct plasmids. Because it has so many plasmids within the narrow size range within which most MAIS plasmids occur, CL26 serves as an excellent source of reference plasmids for use in comparisons of MAIS plasmid profiles during agarose gel electrophoresis. CL26 contains a total of 6 plasmids, and this is the largest number of plasmids that I have observed in any MAIS isolate. Six plasmids were also observed in isolates VA2, VA10, VA390, and 4s.

On the basis of molecular weight, many MAIS isolates appeared to contain identical plasmids. For example, a plasmid of 18.3 Md was observed in 20 isolates, and an 8.8 Md plasmid was observed in 18 isolates (Fig. 1). In order to prove their identity, such commonly observed plasmids should be cloned and used

to probe for sequence homology among various plasmid-positive MAIS isolates. Restriction endonuclease analysis could also be used to test the identity of these plasmids, but this approach is not straightforward for several reasons. For example, because MAIS grow slowly, it is difficult to obtain the quantities of plasmid DNA necessary for extensive restriction analysis. Also, as seen in Figure 1, most MAIS isolates contain 2 or more plasmids. These plasmids must be separated from one another before interpretable restriction endonuclease analyses can be performed.

CHAPTER 3

PLASMID ENCODED MERCURIC REDUCTASE IN *MYCOBACTERIUM SCROFULACEUM*

INTRODUCTION

Previous studies have demonstrated that the increased frequency of recovery of representatives of the Mycobacterium avium, Mycobacterium intracellulare and Mycobacterium scrofulaceum (MAIS) group from water samples collected in the southeastern United States (Falkinham, et al., 1980) correlates with the high frequency of persons showing evidence of prior infection (Edwards et al., 1969) and the high frequency of recovery of mycobacteria of this group from specimens submitted to state tuberculosis laboratories (Good, 1980; Good and Snider, 1982). The wide geographic distribution of members of this group of slow growing, human pathogens throughout the southeastern United States and their ability to grow in natural waters (George et al., 1980) suggested that they should demonstrate phenotypic and genotypic changes which permitted survival in diverse aquatic environments from which they were recovered. Accordingly, we examined the heavy metal resistance of a large number of environmental and clinical MAIS isolates and identified a number of strains which were resistant to 100 μM $HgCl_2$ (Falkinham et al., 1984). Since mercury resistance in both gram-positive (Weiss et al., 1977) and gram-negative (Summers et al., 1978; Summers and Sugarman, 1974) bacteria is due to the presence of plasmids which encode for synthesis of a mercury-volatilizing mercuric reductase, we sought to determine whether the mercury resistance in one environmental MAIS isolate was also due to plasmid encoded mercuric reductase. The results of experiments reported in this paper provide evidence that the resistance to 100 μM $HgCl_2$ of an isolate of M. scrofulaceum is due to the presence of a mercuric reductase

whose appearence correlates with the presence of a plasmid. The identification of this selectable marker on plasmid DNA will allow the development of techniques for genetic analysis of this important group of human and animal pathogens.

MATERIALS AND METHODS

Bacterial isolates. M. scrofulaceum strain W262 was isolated from a brackish surface water sample of the Chester River at Chester, MD near the Chesapeake Bay. Experiments were conducted with cells grown at 30°C in Middlebrook 7H9 medium (BBL, Cockeysville, MD) containing 0.4% (v/v) glycerol (MG broth). The mercury-volatilizing activity was induced by growing strain W262 in MG broth containing 100 μM $HgCl_2$.

The mercury-sensitive strain used in these experiments was obtained after several generations of growth of strain W262 in MG broth followed by plating on nonselective Middlebrook 7H10 (BBL) agar medium containing 0.4% (v/v) glycerol. This spontaneous mercury-sensitive strain (W262C) was obtained from a colony which appeared on this medium and failed to grow in the presence of 100 μM $HgCl_2$.

Mercury volatilization. Whole cell mercury volatilization assays were conducted with log-phase cultures of W262 grown in MG broth plus 100 μM $HgCl_2$. Cells were harvested and resuspended in 1/50 volume of MG broth containing 10 μM $HgCl_2$ and 2 μM $^{203}Hg(NO_3)_2$. All experiments were conducted at 30°C in 16 x 120mm test tubes. Air was gently bubbled through the solution in order to maintain homogeneity and to ensure rapid volatilization of mercury.

Cell-free extracts were prepared by resuspending log-phase cells from 500 ml of MG broth plus 100 μM $HgCl_2$ in 10 ml of 50 mM sodium phosphate buffer (pH 7.0). Cells were disrupted by two passages through a French Pressure Cell (American Instruments Co., Silver Spring, MD) at 20,000 lbs/in^2. After

disruption, the suspension was cleared by centrifugation at 10,000 x g for 10 min. The cell-free supernatant was tested for mercuric reductase activity in a minimal assay mixture (Schottel, 1978) containing 1 ml extract, 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.2 mM $MgSO_4$, 0.5 mg/ml bovine serum albumin, and 2 µM $^{203}Hg(NO_3)_2$. Thiol compounds (final concentration 1 mM), NADPH or NADH (final concentrations 200 µM) were added as indicated in individual experiments.

Plasmid isolation. Plasmid isolation was by the method of Kado and Liu (1981). Mycobacterial strains were grown in 30 ml of MG broth containing 10% OADC (BBL). Upon onset of late log phase, cultures were exposed to D-cycloserine (1 mg/ml) and ampicillin (100 µg/ml) for 18 hours as described by Crawford and Bates (1979). Cells were then harvested and resuspended in 1 ml E buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.9) to which was added 2 ml of lysing solution (3% sodium dodecyl sulfate, 50 mM Tris, pH 12.5) (Kado and Liu, 1981). The lysate was incubated at 60°C for 20 min followed by extraction with 2 volumes of phenol saturated with 0.5 M sodium chloride. Extractions were carried out mechanically at 4 rpm on a New Brunswick roller-drum apparatus (New Brunswick Scientific Co., New Brunswick, NJ). Extracted lysates were cleared by centrifugation at 10,000 x g for 60 min. 50 µl of the aqueous supernatant was mixed with 10 µl of tracking dye (0.25% bromocresol purple in 50% glycerol-0.05 M Tris-acetate, pH 8.2) followed by electrophoresis with a vertical slab apparatus (Hoefer Instuments, San Francisco, CA). Slab dimensions were 16cm x 18cm x 3mm. DNA-grade agarose (0.7%; BioRad Laboratories, Richmond, CA) was dissolved in TEB buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.2) and gels were run for 3 hours at 10 V/cm. After

electrophoresis, gels were stained with a solution of ethidium bromide (0.5 µg/ml for 10 min) and photographed on a short-wave ultraviolet table (Fotodyne, New Berlin, WI) using a Polaroid MP-4 camera.

Molecular weight determination. Plasmids of known molecular weight were isolated from Agrobacterium tumefaciens 1D135 (possessing plasmids of 130 and 260 Md (Kado and Liu, 1981)) and from Mycobacterium intracellulare LR25 (with plasmids of 11.2, 18.3 and 107 Md (Crawford et al., 1981)). Using these plasmids as standards, the molecular weights of the plasmids isolated from strains W262 and W262C were calculated by the method of Meyers et al. (1976).

RESULTS AND DISCUSSION

The plasmid profiles of W262 and its cured derivative W262C are shown in Figure 1. Although W262C contains 3 of the 4 plasmids found in the parental strain, it has lost the 115 Md plasmid (pVT1). This curing occurred spontaneously, but at the low rate of 10^{-4} cured cells per cell per generation. Attempts to increase the rate of curing using neutral acriflavine, mitomycin C, ethidium bromide, or novobiocin were unsuccessful. Concomittant with the loss of pVT1, strain W262C became sensitive to 100 μM $HgCl_2$ (Figure 2). In other mercury-resistant bacteria, resistance is nearly always due to the presence of a plasmid-encoded mercuric reductase which catalyzes the reduction of Hg^{2+} to metallic mercury (Summers and Silver, 1978). Therefore, it seemed likely that the mercury resistance of strain W262 was the result of a mercuric reductase encoded by pVT1. Mercury-volatilizing activity was detected from both whole cells and cell-free extracts of strain W262 grown in the presence of 10 μM or 100 μM $HgCl_2$ (Table I). The sensitivity to 100 μM $HgCl_2$ and lack of mercuric reductase activity in strain W262C grown in the presence of 10 μM $HgCl_2$ (Table 1) is consistent with our belief that the enzyme is encoded by pVT1 in strain W262. The fact that W262C retained the ability to grow in 10 μM $HgCl_2$ is not surprising since a substantial number of clinical and environmental MAIS isolates also grow in the presence of 10 μM $HgCl_2$ (5). The non-specific resistance of MAIS bacteria to high levels of antibacterial compounds is characteristic of these organisms and apparently results from an unusually high permeability barrier (Mizuguchi et al., 1983; Rastogi et al., 1981).

As with other bacterial species (Summers and Sugarman, 1974; Weiss et al., 1977), the mercuric reductase of strain W262 is induced by growth in Hg^{2+}-containing media. Maximum volatilizing activity by either whole cells or cell-free extracts is observed only when those assays are conducted with cells grown in the presence of 100 μM $HgCl_2$ (Table 1).

Bacterial mercuric reductases are cytoplasmic enzymes (Schottel, 1978), and the enzyme from strain W262 is associated with the soluble fraction of cell-free extracts. Seventy percent of the original enzyme activity of crude cell extracts was present in the supernatant following centrifugation at 145,000 x g for 60 min (data not shown). The activity of the enzyme was completely dependent on NAD(P)H and there was essentially no difference in activity when either NADH or NADPH were supplied as electron donors (Table 1). Plasmid-encoded reductases from other species often show activity in the presence of either cofactor, but activity is usually greater with NADPH than with NADH (Summers and Silver, 1978). However, there are the reports of mercury-volatilization by a soil pseudomonad (Furukawa and Tonomura, 1971) and a Streptomyces spp. (Silver, 1983) whose mercuric reductases use either cofactor equally as we have observed.

Mercuric reductases from gram-positive microorganisms (such as Staphylococcus spp. and Bacillus spp.) are inactivated by temperatures above 60°C (Olson et al., 1982). Incubation for 10 min 63°C completely inactivated the mercuric reductase from strain W262 (data not shown). Additionally, the enzyme retained activity after several freeze-thaw cycles (data not shown) and in this respect it resembles the reductase from Thiobacillus ferrooxidans (Olson et al., 1982).

Unlike previously studied mercuric reductases (Schottel, 1978; Summers and Silver, 1978; Weiss et al., 1977), the activity of the enzyme in freshly prepared extracts of strain W262 was not dependent on exogenous thiol compounds. Addition of ß-mercaptoethanol at concentrations necessary for the activity of reductases from other species (Olson et al., 1982), had little or no effect on the enzyme from M. scrofulaceum (Table 1). In other species, maximum reductase activity occurs when the reduced thiol is present at concentrations nearly 100-times in excess of the substrate Hg^{2+} (Schottel, 1978; Summers and Sugarman, 1974). This large molar excess of sulfhydryl compound implies that the actual substrate for the reductase may be a thiol (or dithiol) adduct of mercury and not ionic mercury (Summers and Silver, 1978). The fact that the reductase from M. scrofulaceum strain W262 is active in the absence of exogenous thiol compounds may indicate that other compounds present in cell extracts from this strain may react with Hg^{2+} and thereby render it a suitable substrate for the enzyme.

M. scrofulaceum is closely related to two other mycobacterial species; M. avium and M. intracellulare (Wolinsky, 1979). Although plasmids have been isolated from these two latter species (Crawford et al., 1981; Mizuguchi et al., 1981), this is the first report of plasmids in an environmental isolate of M. scrofulaceum. Though there is evidence that mycobacterial plasmids encode for restriction-modification (Crawford et al., 1981) and are involved in colonial morphology variation (Mizuguchi et al., 1981), neither is a useful marker for genetic selection. The discovery of a plasmid-encoded mercuric reductase in M. scrofulaceum suggests that this trait will serve as an important marker in the genetic analysis of these species as well as in epidemiological studies.

TABLE 1. Characterization of mercury volatilization by cells and cell-free extracts of *Mycobacterium scrofulaceum* strains W262 and W262C.

Assay	Strain	Growth Conditions[a]	Nucleotide or Thiol Addition[b]	Volatilization Rate[c]
Whole Cells	W262	100.	none	50.
	W262	10.	none	16.
	W262C	10.	none	<1.
	W262	0	none	<1.
Cell-free Extract	W262	100.	none	<1.
	W262	0	NADPH or NADH + β-ME	<1.
	W262C	10.	NADPH or NADH + β-ME	<1.
	W262	100.	NADH	44.
	W262	100.	NADPH	42.
	W262	100.	NADPH + β-ME	41.

[a] Concentration (μM) of $HgCl_2$ in MC broth in which cells of either strain were grown to late log-phase.

[b] Cofactors added to reaction mixture at 200 μM NAD(P)H and 1 mM β-Mercaptoethanol.

[c] pmoles ^{203}Hg volatilized per 10^8 cells per min

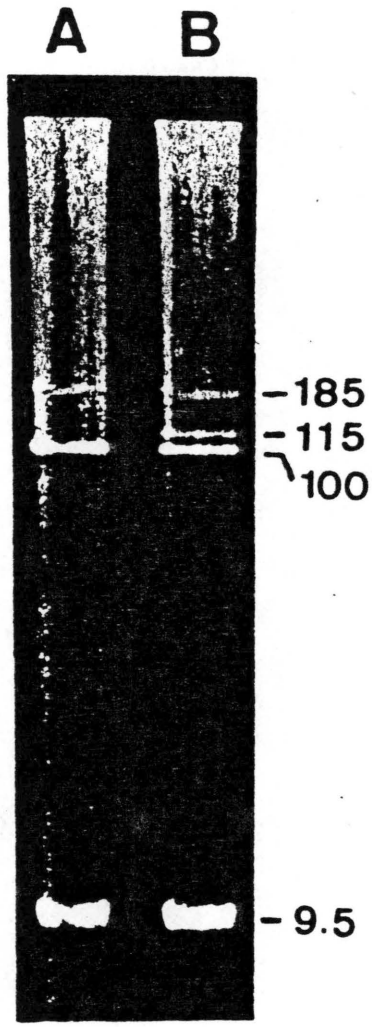


FIG. 1. Agarose gel patterns of plasmids from strains W262C(A) and W262 (B). Numbers on the right represent molecular weights (in megadaltons) of plasmids calculated by comparing their mobility with those of standards (see text).

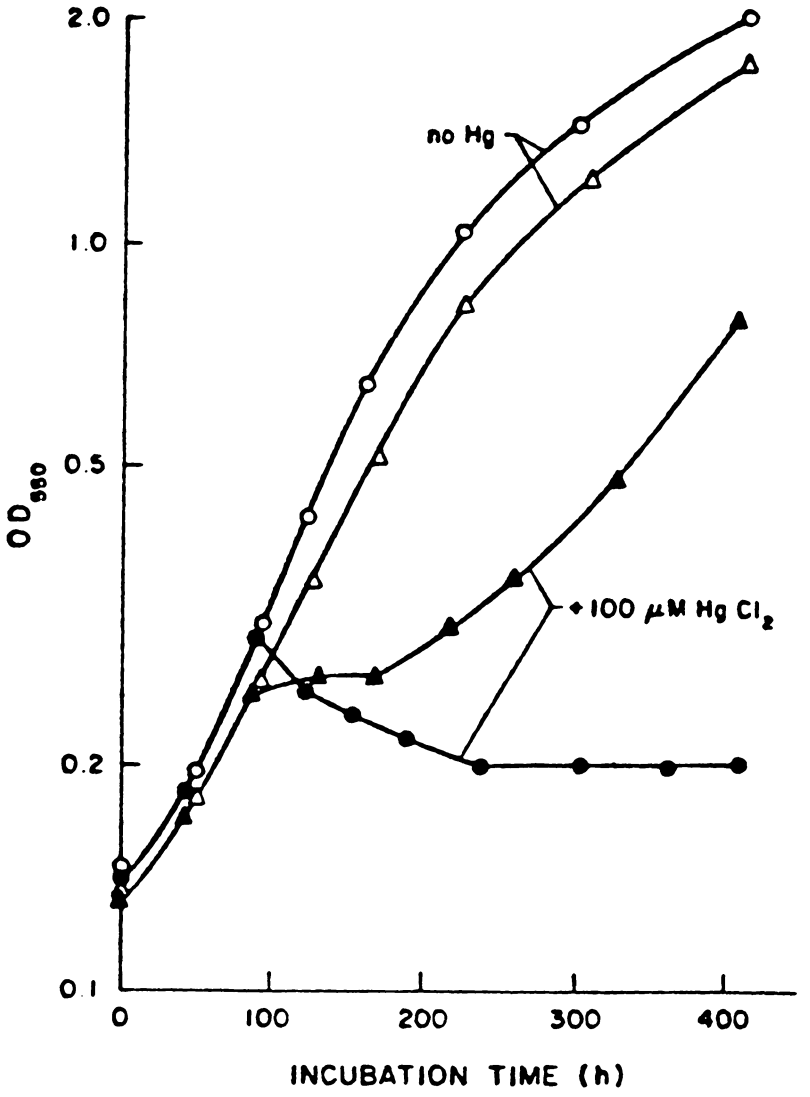


FIG. 2. Growth of strains W262 (△,▲) and W262C (○,●) exposed (▲,●) or not exposed (△,○) to 100 μM $HgCl_2$. Log-phase cells grown in MG broth were diluted into 30 ml fresh MG broth in 250 ml sidearm flasks and incubated with slow shaking at 30°C. Initial inocula were approximately 5 x 10^7 colony forming units/ml (optical density at 550 nm of 0.15). After 100 hr of incubation, $HgCl_2$ was added to a final concentration of 100 μM to one of two duplicate flasks.

CHAPTER 4

CHARACTERIZATION OF MERCURY RESISTANT HUMAN AND ENVIRONMENTAL ISOLATES OF THE *MYCOBACTERIUM AVIUM-INTRACELLULARE-SCROFULACEUM* COMPLEX

INTRODUCTION

Human infection by bacteria of the Mycobacterium avium-intracellulare-scrofulaceum (MAIS) complex is believed to result from contact with environmental sources of these pathogens (Wolinsky, 1979). Some of the environments from which MAIS have been isolated are known to be polluted with heavy metals (Villa and Johnson, 1974; Johnson and Villa, 1976). Therefore, we thought that it should be possible to identify and isolate heavy metal resistant human and environmental MAIS isolates. Accordingly, we screened a collection of human and environmental MAIS isolates for resistance to a variety of heavy metal salts and oxyanions (Falkinham et al., 1984). From this study, we identified a number of isolates which were resistant to 100 uM $HgCl_2$. In one of these isolates (M. scrofulaceum strain W262), we showed that mercury resistance resulted from the synthesis of a heat-labile, NAD(P)H-dependent mercuric reductase (Meissner and Falkinham, 1984). Additionally, we showed that the mercuric reductase was encoded by a 115 megadalton (Md) plasmid in W262. In this report, we extend these findings to show that 115 Md plasmids are present in several mercury resistant human and environmental MAIS isolates obtained from diverse locations. We also present evidence which suggests that, in some mercury resistant isolates, the mercuric reductase gene may be located on plasmids of diverse sizes or may even be located on the mycobacterial chromosome.

MATERIALS AND METHODS

The 28 mercury resistant isolates were selected for this study based on their abilities to grow to an optical density greater than 0.5 (at 550nm) in Middlebrook 7H9 broth (BBL Microbiological Systems, Cockeysville, MD) containing 0.4% (w/v) glycerol and 100 uM $HgCl_2$. The procedures for plasmid isolations and whole cell and cell free volatilization assays have been described by us previously (Meissner and Falkinham, 1984). The standard (control) reaction condition for cell free volatilization assays was the following: 1 ml of cell free extract in a minimal assay mixture (Schottel, 1978) containing 1 mM B-mercaptoethanol and 200 uM each of NADH or NADPH.

RESULTS AND DISCUSSION

The species identifications, source, and volatilization rates of the 28 mercury resistant isolates used in this study are listed in Table 1. Sixteen (57%) of the isolates were M. avium-intracellulare, and the remaining 12 (43%) were M. scrofulaceum. Volatilization of ^{203}Hg was detected in whole cell assays of each of the 28 isolates. Volatilization rates ranged from 5 to 55 pmole ^{203}Hg volatilized per 10^8 cells per min. These rates are 3- to 4- orders of magnitude less than volatilization rates obtained with mercury resistant isolates from other genera (Summers and Sugarman, 1974; Olson et al., 1982). Because of these low rates, and because of the extremely slow growth rate of MAIS bacteria, we were unable to obtain quantities of cell extracts sufficient for the characterization of features such as V_{max}, K_m, pH optima, thiol requirements (Olson et al., 1982). However, we were able to obtain some volatilization rates on cell free extracts of six of the 28 isolates. These data are presented in Table 2. Perhaps the most interesting finding was that 4 of the 6 isolates contained mercuric reductases that were considerably more heat stable than the reductases of strains W262 and MD1. In these two strains, approximately 90% of the reductase activity is abolished when cell extracts are heated to 63°C for 10 min. Yet, in the other 4 strains, at least 50% of the activity remains even after 10 min at 73°C. And, in one strain (VA5), 50% of the original activity is still present after heating for 10 min at 83°C. Heat lability is considered to be a property of the mercuric reductases of gram positive bacteria (Olson et al., 1982), while heat stability is characteristic of the reductases from gram

negative bacteria (Summers et al., 1974). Thus, on the basis of heat stability, MAIS isolates appear to contain at least two distinct reductase enzymes. However, with respect to other characteristics, the mercuric reductases of these 6 isolates appear similar to one another. For example, they are freeze-thaw stable and retain activity in the absence of B-mercaptoethanol (Table 2).

Bacterial mercuric reductases are nearly always encoded by genes residing on plasmids (Robinson and Tuovinen, 1984), and often these genes lie within the domains of transposable elements (Tanaka et al., 1983). Therefore, we were not surprised to find that 24 of the 28 mercury resistant MAIS isolates contained one or more plasmids (Figure 1). Previously, we have shown that the reductase of strain W262 is encoded by a 115 Md plasmid, pVT1 (Meissner and Falkinham, 1984). A 115 Md plasmid was observed in 15 (54%) of all isolates (Fig. 1), and thus it appears as if pVT1 is widely disseminated among mercury resistant human and environmental MAIS isolates. For example, pVT1 was observed in a Canadian human isolate (TMC 1323) and in an environmental isolate from Miami, Florida (W240). Of course, proof that these 115 Md plasmids are in fact identical to pVT1 must await development of techniques for isolation of a single mercury-resistance plasmid in sufficient amounts for Southern blot analysis.

Interestingly, not all mercury resistant MAIS isolates contained a 115 Md plasmid (Fig. 1). A 160 Md plasmid was detected in four isolates which had no other plasmids in common. Thus, the reductase gene appears to be encoded by a 160 Md plasmid in these isolates. And, in five isolates, the reductase gene may be encoded by small (<30 Md) plasmids. If the reductase determinant resides on such a small plasmid, it should be possible to clone it and localize the reductase

gene on a fragment small enough to serve as a probe for determining whether homologous sequences are present in other plasmids such as the 160 Md plasmids and pVT1. Additionally, chromosomal restriction digests from the 4 plasmidless isolates could be probed in order to determine if reductase genes are encoded by the mycobacterial chromosome. If such an analysis reveals homology among the mercuric reductase determinants of all mercury resistant MAIS isolates, then this will indicate that the differences in heat stability of the mercuric reductases from these isolates are not a result of significant sequence divergence. Recent evidence has revealed that the mercuric reductase determinants of Enterobacteriacae are homologous (Tanaka et al., 1983). Thus, it would be surprising to find substantial sequence divergence among reductase determinants of the closely related M. avium, M. intracellulare and M. scrofulaceum species.

Table 1. Mercury resistant human and environmental MAIS isolates used in this study.

Isolate	Species[a]	Rate[b]	Source[c]	Location[d]	Reference
NY 13541	MAI	29	H	New York	this study
NY 93191	MAI	31	H	"	"
MD 1	MAI	27	H	Maryland	"
MD 2	MAI	21	H	"	"
MD 17	MS	24	H	"	"
MD 21	MS	10	H	"	"
MD 23	MS	14	H	"	"
VA 5	MAI	55	H	Virginia	"
VA 9	MAI	15	H	"	"
VA 18	MAI	41	H	"	"
VA 19	MAI	19	H	"	"
VA 22	MAI	16	H	"	"
Va 280	MAI	11	H	"	"
SC 2	MAI	9	H	South Carolina	"
SC 7	MAI	10	H	"	"
TMC 1321	MS	20	H	TMC[e]	( )[f]
TMC 1323	MS	23	H	TMC	( )[g]
W 39	MS	36	W	Hopewell,VA	this study
W 240	MS	16	W	Miami, FL	"
W 262	MS	50	W	Chester R., MD	( )[h]
CL 25	MS	12	CL	Virginia	this study
CL 26	MS	13	CL	"	"
CL 28	MS	5	CL	"	"
CL 29	MAI	13	CL	"	"
DE 10	MAI	20	W	Delaware Bay	"
DE 17	MAI	17	W	Delaware Bay	"
S 5	MAI	22	A	Richmond, VA	"
SR 666	MS	23	W	Savannah River	"

[a] MAI = M. avium-intracellulare; MS = M. scrofulaceum.

[b] pmoles ^{203}Hg volatilized per 10^8 cells per min.

[c] H,W,CL, and A refer to human, water, chicken litter, and aerosol MAIS isolates.

[d] Location from which isolates were obtained.

[e] TMC = Trudeau Memorial Collection.

[f] Wayne and Lessel, (1969).

[g] Prissick and Masson, (1956).

[h] Meissner and Falkinham, (1984).

Table 2. Cell free mercury volatilization data for six MAIS isolates.

	Percent volatilization relative to control[a]							
	pre-treated extracts[b]					nucleotide/thiol[c]		
Isolate	63°	68°	73°	83°	Frozen	NADH	NADPH	-BMerc
W262	11	-[d]	-	-	91	66	75	90
MD 1	14	-	-	-	87	38	68	56
MD 2	153	-	64	-	67	77	130	-
MD 17	118	134	50	-	82	90	82	75
VA 5	52	99	91	50	35	10	75	85
CL 29	57	110	160	-	94	65	28	75

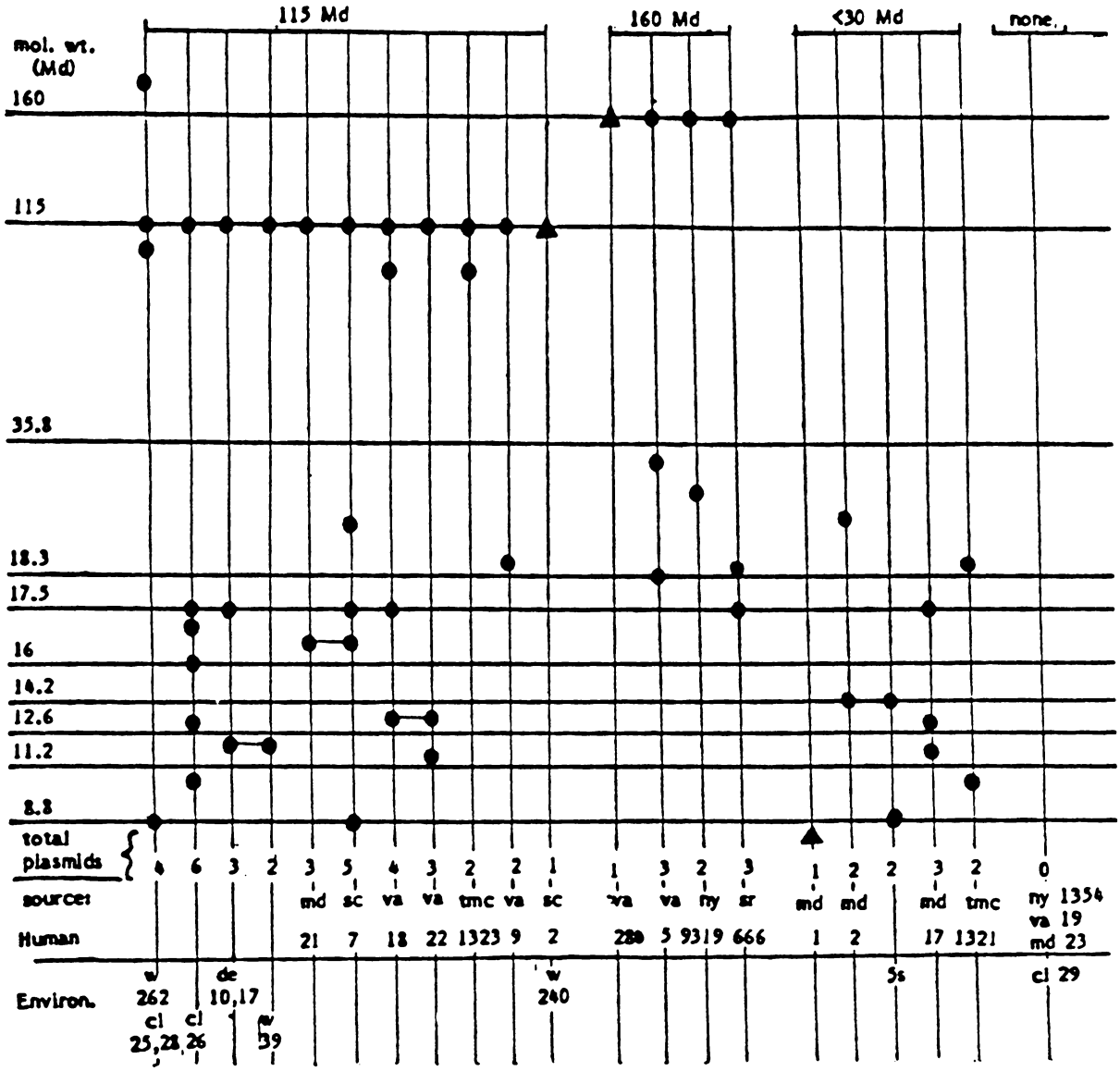
[a]Values represent the percent of ^{203}Hg volatilized relative to the control reaction mixture (see text).

[b]Pre-treated refers to the fact that cell free extracts were heated for 10 min at the indicated temperature prior to determination of ^{203}Hg volatilization; Frozen indicates that cell free extracts were freeze-thawed once prior to determination of ^{203}Hg volatilization.

[c]Instead of adding 200 uM each of NADH and NADPH (control mixture), only the indicated cofactor was added; -BMerc indicates that B-mercaptoethanol was omitted from the control mixture.

[d]"-" indicates that the value was not determined.

Figure 1. Plasmid profiles of mercury resistant MAIS isolates[a].



[a]Black dots represent plasmids observed during agarose gel electrophoresis. Vertical lines represent the plasmid profile of the MAIS isolate whose number and letter designation are given at the base of each line. Horizontal lines show the positions of plasmids whose molecular weights are given at the left hand side of each line. For isolates containing only one plasmid, triangles were used instead of dots.

CHAPTER 5

PLASMID ANALYSIS AS AN EPIDEMIOLOGIC TOOL IN STUDIES OF THE *MYCOBACTERIUM AVIUM-INTRACELLULARE-SCROFULACEUM* COMPLEX

INTRODUCTION

The Mycobacterium avium, M. intracellulare, and M. scrofulaceum (MAIS) complex consists of a heterogenous group of slow growing, opportunistic pathogens. The greater frequency of isolation of MAIS bacteria from natural waters of the southeastern compared to the northeastern United States (Falkinham et al., 1980; Brooks et al., in press) correlates with the high frequency of persons showing evidence of prior infection (Edwards et al., 1969) and the high frequency of recovery of mycobacteria of this group from specimens submitted to state tuberculosis laboratories (Good, 1980; Good and Snider, 1982). In addition to their isolation from water, these organisms have also been detected in a variety of other environmental samples such as dust (Tsukamura et al., 1974), soil (Wolinsky and Rynearson, 1968), and aerosols (Wendt et al., 1980). Despite these findings, the mechanisms by which environmental sources of MAIS colonize and infect and cause disease remain unclear (Wolinsky, 1979). Lack of adequate epidemiological markers has thwarted efforts to clarify this problem. Serotype is the most commonly used marker (Codias and Reinhardt, 1979; Tsang et al., 1983), yet it suffers from certain limitations (Crawford et al., 1981c). For example, a significant percentage of the MAIS isolates which are submitted for serotypic analysis are either untypable or autoagglutinate (Brennan et al., 1982). Likewise, despite initial studies which indicate that MAIS isolates are suitable for phage-typing (Crawford et al., 1981c), a formal typing scheme has yet to be developed.

In other bacterial genera, extrachromosomal (plasmid) DNA content has proven to be a useful addition to traditional epidemiological markers such as serotype and phage-type (McGowan et al., 1979; Parisi and Hecht, 1980; Riley et al., 1983; Farrar, 1983). Prompted by the report of plasmids among MAIS clinical isolates (Crawford et al., 1981a), we initiated a study to test the feasibility of using plasmid profiles as epidemiological markers. Our first objectives were to determine the frequency of plasmids among large numbers of human and environmental isolates, and to examine the stability of plasmids under conditions encountered in routine laboratory culture and decontamination procedures. The importance of this latter study is underscored by the fact that clinical and environmental samples are often exposed to NaOH to aid recovery of these slow growing pathogens. We present data which indicates that MAIS plasmid profiles are not altered following NaOH exposure. Because of their stability under these conditions and the high frequency of plasmids among clinical isolates, we believe that plasmid profiles will be useful markers in epidemiological studies of MAIS-associated diseases. Interestingly, of the environmental isolates we examined, aerosol isolates most closely resembled human isolates. This observation supports the belief that naturally occuring aerosols may be an important source of the mycobacteria responsible for human disease (Wendt et al., 1980;Parker et al., 1983). Additionally, this finding suggests that plasmids may encode for functions affecting the movement of MAIS in the environment and for functions involved in pathogenesis.

MATERIALS AND METHODS

Human Isolates. The human MAIS isolates used in this study were primary isolates obtained predominantly from the sputum of patients with suspected pulmonary infection (Table 1, Chapter 1). We obtained the isolates from the state tuberculosis laboratories of New York, Maryland, Virginia, South Carolina, and Georgia. The isolates were mailed to our laboratory on Lowenstein-Jensen slants. The majority of isolates were assigned to the M. avium-intracellulare complex. The remainder of the isolates were M. scrofulaceum.

Environmental Isolates. The recovery of water (Falkinham et al., 1980), soil and sediment (Brooks et al., in press), and aerosol (Wendt et al., 1980) MAIS isolates is described in the references indicated. Dust isolates were obtained with a six-stage Andersen sampler (Andersen, 1958) as described by Wendt et al. (1980).

Plasmid Isolation. All MAIS isolates were grown at 30°C in Middlebrook 7H9 medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.4% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) enrichment. We refer to this medium as MGE broth. The OADC enrichment was prepared in the following manner: sodium chloride (0.85% w/v), catalase (0.03 g), bovine serum albumin (50 g), and dextrose (20 g) were dissolved in 1000 ml distilled water at ambient temperature. The solution was then transferred to a cold (8°C) room and oleic acid (0.6 ml) was added. The solution was mixed at medium speed on a magnetic stirring device until the oleic acid went into solution (usually about 2 hours). The solution was then filter sterilized and

dispensed into sterile screw cap tubes in 20 ml aliquots. Tubes were then held indefinitely at 8°C in the dark. The procedure used for the screening and molecular weight determination of MAIS plasmids has been described by us elsewhere (Meissner and Falkinham, 1984).

Restriction endonuclease digestion. Purification of plasmid DNA for restriction endonuclease digestion was performed as described by Kado and Liu (1981). Restriction endonucleases were from Bethesda Research Labs (BRL, Gaithersburg, MD) and restriction digests were carried out in accordance with instructions provided by BRL. Vertical gel electrophoresis was performed as we have described previously (Meissner and Falkinham, 1984).

NaOH Treatment. Ten plasmid-positive and 10 plasmidless isolates (5 human and 5 environmental isolates in each case) were selected for a study to determine their relative rates of survival when subjected to a standard decontamination treatment (Falkinham et al., 1980; Brooks et al., in press). Each isolate was grown in 10 ml MGE broth to mid-log phase (1×10^8 cfu/ml). One ml was transferred to each of 2 sterile tubes which were then centrifuged at 8,000 x g for 20 min at ambient temperature. The supernatant from both pellets was discarded; one pellet was suspended in 10 ml sterile water (control treatment), while the other pellet was suspended in 10 ml 1% (w/v) sterile NaOH. Both pellets were then processed as described elsewhere (Falkinham et al., 1980; Brooks et al., in press). At the conclusion of the decontamination procedure, bacterial suspensions were spread-plated on Middlebrook 7H10 (BBL) containing 10% OADC enrichment. Plates were incubated at 30°C for 2 weeks, at which time the results were recorded (see Table 2). Additionally, in order to determine what effect NaOH exposure might have on plasmid profiles, single

colonies from spread plates of plasmid-positive isolates exposed to NaOH were inoculated into 10 ml of MGE broth. A total of 25 colonies were examined; 5 colonies were picked from plates following exposure of 5 different plasmid-positive isolates to NaOH. The cultures were incubated at 30°C and plasmid isolations were performed as described previously (Meissner and Falkinham, 1984).

Statistical analysis. Means were compared by Student's t-test, and proportions were compared by the chi-square procedure. P=0.05 was considered significant.

RESULTS

The frequency of plasmid-positive isolates among human (n=131) and environmental (n=196) MAIS isolates is summarized in Table 1. We separated the M. avium-intracellulare (MAI) results from M. scrofulaceum (MS) results because of the greater clinical significance of MAI bacteria. Because MS is infrequently associated with human disease (Wolinsky, 1979), we received predominantly MAI human isolates from the state tuberculosis laboratories. Plasmids were detected in 64 of 116 human MAI isolates and in 9 of 15 human MS isolates. Thus, the frequency of plasmids in both groups was nearly identical (X^2= 0.1, P>0.8).

Plasmids were common among human isolates, regardless of the state from which they were obtained (Table 1). Plasmids were also common among aerosol isolates. In contrast, plasmids were infrequently encountered among soil, sediment, dust, and water MAIS isolates. Overall, the frequency of plasmids was greater among water isolates than among soil, sediment, or dust isolates. However, plasmids were detected in only 20% of the 50 water MAI isolates, compared to 75% of the 16 aerosol MAI isolates (X^2= 16.8, P<0.001). Thus, plasmids are significantly more frequent among aerosol MAI isolates than among water MAI isolates.

The plasmid profiles of human and environmental isolates were so heterogenous that it was not possible to correlate any particular plasmid profile definitively with a geographic region. However, plasmids in the 20 to 30 Md size range were most common among human and aerosol isolates from the Virginia/James River region compared to the other 2 regions. The plasmid

profiles of some of these human and aerosol isolates are shown in Figure 1. Note that, although some of these isolates appear to contain identical plasmids (on the basis of molecular weight), isolates with identical profiles are not shown. This is because we rarely encountered identical profiles among any group of isolates. However, we observed that the plasmid profiles of aerosol isolates more closely resembled those of human isolates than did the profiles of any other environmental isolates. For example, plasmid-positive water, soil, sediment, and dust MAIS isolates generally contained only a single plasmid (data not shown). In contrast, plasmid-positive aerosol and human isolates usually contained 2 or more plasmids (Figure 1; unpublished observation). Additionally, the 11.2, 14, 16.9, and 18.3 Md plasmids (Figure 1) were common among aerosol and human isolates but were rarely detected in isolates from the other environmental sources (data not shown).

Because we encountered few isolates with identical plasmid profiles and most plasmid-positive MAIS isolates contained 2 or more plasmids (unpublished observation), we were only able to perform restriction endonuclease digestions on a few MAIS isolates. For example, we observed a single 16.5 Md plasmid in two M. scrofulaceum isolates. These isolates were a human isolate (TMC1312) and a Savannah River water isolate (SR353). Preparations of plasmid DNA from each isolate comigrated as a single band during agarose gel electrophoresis . Additionally, both plasmids yielded identical patterns of fragmentation following digestion with restriction endonuclease XhoI (Figure 2).

The aerosol isolates were obtained when aerosolized droplets contacted petri plates containing appropriate growth media (see Wendt et al., 1980). Thus, the aerosol isolates were the only environmental isolates obtained without

having been subjected to NaOH decontamination. Since some decontamination treatments have been observed to eliminate plasmids (Hill and Carlisle, 1981), we wondered whether this might account for the lower frequency of plasmids among non-aerosol environmental isolates. Also, if the MAIS bacteria that carry plasmids are more NaOH-sensitive than plasmidless MAIS, decontaminated samples would be artificially enriched for plasmidless mycobacteria. In Table 2 we present the results of a study designed not only to test the effect of a standard NaOH decontamination treatment (Falkinham et al., 1980) on plasmid profiles, but also to determine the NaOH sensitivity of plasmid-positive and plasmidless MAIS isolates. There was no significant difference in the NaOH sensitivity of plasmid-positive versus plasmidless isolates ($T=0.82$, $P>0.4$). Additionally, no plasmid profile alterations were detected following exposure of plasmid-positive isolates to NaOH (Table 2).

Table 1. Frequency of Plasmid-Positive Human and Environmental Isolates of *Mycobacterium avium-intracellulare* and *M. scrofulaceum*

Isolate Origin[a] (Number)[b]	Percent Containing Plasmids	
	M. avium-intracellulare	*M. scrofulaceum*
Tombigbee River		
water (3;0)	33	NT[c]
soil (18;0)	6	NT
GA & SC/Savannah River		
human (34;0)	47	NT
water (7;10)	<14	30
soil (33;9)	6	<11
VA/James River		
human (49;0)	55	NT
aerosol (16;0)	75	NT
water (26;11)	12	18
sediment (4;12)	<25	<8
soil (5;1)	<20	<100
dust (11;3)	<9	33
MD/Chesapeake & Delaware Bays		
human (14;11)	79	55
water (10;12)	50	50
NY/assorted[d]		
human (19;4)	53	75
water (2;3)	50	<33
TOTAL		
human (116;15)	55	60
environment[e] (137;59)	18	20
aerosol (16;0)	75	NT
water (50;34)	20	32
soil (56;10)	5	<10
sediment (4;12)	<25	<8
dust (11;3)	<9	33

[a] headings are listed as follows: state from which human isolates were obtained/river (or bay) at (or near which) environmental isolates were obtained

[b] (number of *M. avium-intracellulare* tested; number of *M. scrofulaceum* tested)

[c] none tested

[d] water isolates were from an assortment of locations in the New York region

[e] environment = summary of results for all compartments tested

Table 2. Influence of NaOH exposure on plasmid-positive and plasmidless MAIS isolat

Isolates (number)[a]	Percent survival following NaOH exposure[b]	Percent surviving isolates with altered profiles
Plasmid-positive (10)	25±15	<4[c]
Plasmidless (10)	31±16	NA[d]

[a] number of isolates tested.
[b] values are given as the mean percent survival plus or minus the standard deviation. Data from control treatments is not shown because all isolates had >95% survival.
[c] none of the 25 colonies tested had any alteration of their plasmid profiles following exposure to NaOH.
[d] not applicable

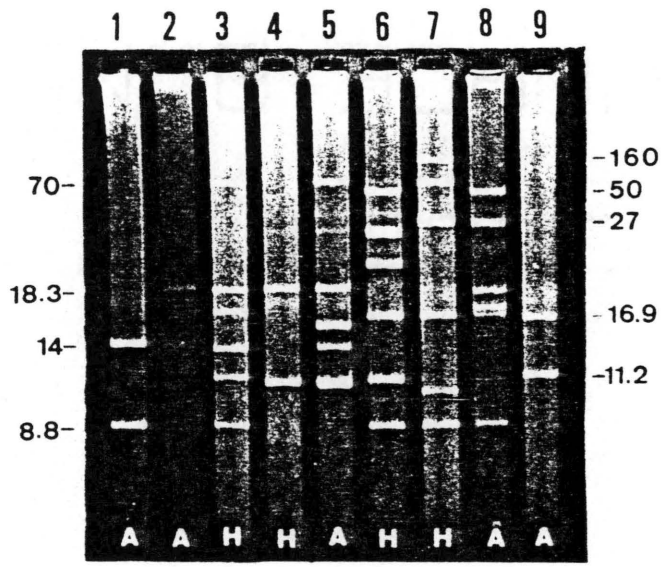


Figure 1. Agarose gel electrophoresis of plasmid DNA isolated from Virginia human and environmental (aerosol) MAIS isolates. Lanes marked A are aerosol isolates, those marked H are isolates from humans. Lane 1, 5s; lane 2, 7s; lane 3, VA 310; lane 4, VA 12; lane 5, 18s; lane 6, VA 2; lane 7, VA 10; lane 8, 4s; lane 9, 12s. Migration was from top to bottom. Horizontal electrophoresis was carried out at 4 V/cm of gel length for 15 hr. The gel dimensions were 0.4 x 20 x 25 cm, with an agarose concentration of 0.8%. Numbers to the right and left represent molecular weight (in megadaltons).

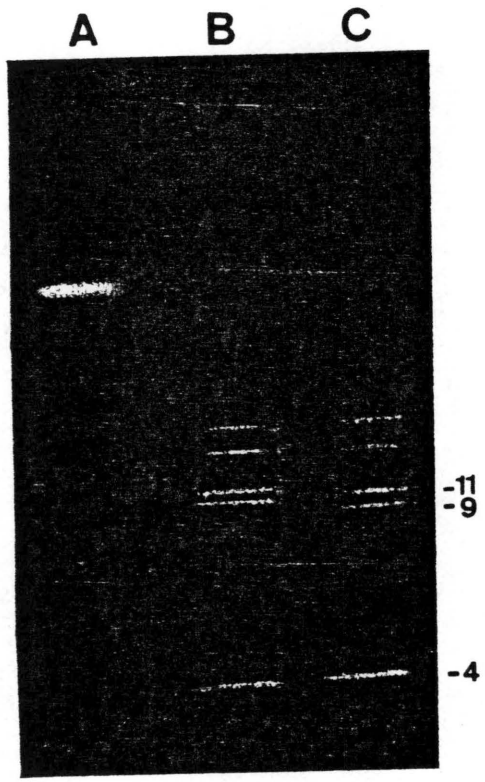


Figure 2. Restriction endonuclease analysis of plasmids from M. scrofulaceum isolates TMC1312 and SR353. Lane A, comigration of plasmid DNA purified from each isolate; Lanes B and C represent XhoI restriction digests of plasmid DNA purified from isolates TMC1312 and SR353, respectively. Unlabelled bands in lanes B and C represent incompletely digested fragments. Complete digestion yields three fragments of 4, 9 and 11 kb.

DISCUSSION

Prompted by the report of plasmids in MAIS clinical isolates (Crawford et al., 1981a), we initiated a study to test the feasibility of using MAIS plasmid profiles as epidemiological markers. Obviously, if plasmids are to serve as useful markers, it must be shown that they are quite common among clinical isolates. We have demonstrated that plasmids are frequently present in human MAIS isolates. We have also shown that MAIS plasmid profiles are remarkably heterogenous (Figure 1). Although we have a number of isolates which share similar plasmids on the basis of molecular weight, we rarely encountered two isolates with identical profiles. We have identified a number of plasmids which are common among MAIS isolates (for example, the 8.8 Md plasmid shown in Fig. 1), and we hope to clone some of these plasmids. Once cloned, these plasmids could be used as probes to test for the presence of homologous sequences among a variety of plasmid-positive MAIS isolates. To date, only a single MAIS plasmid has been cloned (Crawford and Bates, 1984). Restriction endonuclease analysis could be used to test the identity of various MAIS plasmids, but because most isolates contain more than one plasmid (unpublished observation), this approach is not straightforward. Also, because some MAIS plasmids encode restriction-modification enzymes (Crawford et al., 1981b), they may resist digestion by some restriction enzymes (Crawford and Bates, 1984). However, we were able to demonstrate the identity of a 16.5 Md plasmid isolated from a human (isolate TMC1312) and an environmental (isolate SR353) isolate of M. scrofulaceum using restriction endonuclease digestion. We obtained SR353 from

the Savannah River in 1980 (R. W. Brooks, M.S. Thesis, Virginia Polytechnic Institute and State University), whereas the human isolate was deposited in the Trudeau Memorial Collection nearly 30 years ago (Prissick and Masson, 1956). These findings provide further evidence (see below) that similar plasmids are present in MAIS isolated from diverse locations.

In other epidemiological studies, the identity of plasmids has been determined by examining plasmid-encoded phenotypes such as resistance to various antibiotics (Tompkins et al., 1980; Shlaes et al., 1983; Markowitz et al., 1983). Unfortunately, efforts to correlate MAIS drug resistance with specific plasmids have failed (Mizuguchi et al., 1983), and thus the vast majority of MAIS plasmids are currently considered cryptic. However, we have determined that a 115 Md plasmid (pVT1) encodes mercury resistance in M. scrofulaceum strain W262 (Meissner and Falkinham, 1984), and we have since identified a number of mercury resistant human and environmental M. avium-intracellulare and M. scrofulaceum isolates (Falkinham et al., 1984). Most of these isolates contain a 115 Md plasmid (unpublished observations). Based on these preliminary findings, it appears as if pVT1 may be an example of a plasmid shared by both human and environmental MAIS isolates. Our efforts to prove the identity of these 115 Md plasmids have been hampered by a number of factors, including the lack of a suitable probe for Southern blot analysis.

Because human infection by MAIS bacteria is believed to result from contact with environmental sources of these pathogens (Wolinsky, 1979), we screened for plasmids among our collection of dust, soil, sediment, water, and aerosol MAIS isolates. MAIS bacteria are pulmonary pathogens (Wolinsky, 1979), and recent reports have demonstrated that MAIS are present in naturally

occurring aerosol droplets of sufficiently small size to penetrate the human lung (Gruft et al., 1975; Wendt et al., 1980; Parker et al., 1983). On the basis of plasmid content, we found that aerosol isolates more closely resembled human isolates than did isolates from the other environmental sources we examined. Plasmids were common among both human and aerosol MAIS isolates, but they were encountered less frequently among non-aerosol environmental isolates. Additionally, the plasmid profiles of human and aerosol isolates were similar. These observations provide further evidence that MAIS in natural aerosols may contribute significantly to human colonization and disease.

We determined that the percent survival of plasmid-positive and plasmidless human and environmental MAIS isolates was similar following a standard NaOH decontamination treatment. We also found that this treatment had no effect on the plasmid profiles of the human and environmental MAIS isolates we tested. Although the plasmid-positive and plasmidless isolates used in this study were not isogenic, our findings suggest that NaOH decontamination treatments have little effect on the recovery of plasmid-positive MAIS isolates.

Struck by the high frequency of plasmids among human and aerosol MAIS isolates, we wonder whether these plasmids are simply cryptic, or if they might be involved in determining characteristics which affect the movement of MAIS in both animate and inanimate surroundings. For example, recent findings (George and Falkinham, submitted) indicate that specific mycobacterial cell surface determinants may be involved in both aerosolization (Parker et al., 1983) and epithelial cell invasiveness (Mapother and Songer, 1984). As seen in Table 1, we presently have only a limited collection of aerosol isolates. We are currently working on methods designed to facilitate the collection of naturally

occuring aerosols from various bodies of water, and we expect that the isolation and characterization of greater numbers of aerosol isolates will shed more light on this important problem.

CHAPTER 6

CONCLUDING REMARKS

As discussed in Chapter 1, the first objective of this study was to determine whether plasmids were present in human and environmental MAIS isolates. To do this, I first had to develop a suitable plasmid isolation procedure. Fortunately, by the time this study was initiated, Crawford had developed a drug treatment which allowed MAIS isolates to be gently lysed by sodium dodecyl sulfate (Crawford et al., 1981a) and Kado and Liu had developed a simple and rapid procedure for the isolation of large and small plasmids (Kado and Liu, 1981).

Although Crawford's drug treatment procedure was adequate for the plasmid screenings undertaken in this study, the method has certain drawbacks. For example, as discussed in Chapther 1, MGE broth presently appears to be the only medium in which MAIS can be grown for plasmid isolations. This fact imposes certain limitations on MAIS plasmid isolations. For example, unlike many other bacterial genera which can be lysed by treatment with lysozyme and sodium dodecyl sulfate (Holmes and Quigley, 1981; Eckhardt, 1978), single mycobacterial colonies cannot be directly tested for plasmids because of their resistance to lysis by these treatments. Instead, the colonies must first be grown in MGE broth. This growth can be very slow because even after weeks of growth the MAIS colony used for inoculum is often quite small; and I have observed that when MAIS broth cultures are inoculated with low numbers of bacteria ($<10^4$ viable colony forming units per ml), several weeks are required before significant growth occurs. Also, the requirement that a MAIS isolate be grown in MGE broth prior to plasmid isolation means that selective pressures cannot be maintained on the bacterial population. For example, heavy metals

such as mercury and cadmium cannot be added to MGE broth because these metals will complex with bovine serum albumin. Therefore, all of the plasmids that I detected in this study were present despite growth of the host cells in nonselective MGE broth. In other bacterial genera (Day, 1982; Novick, 1969), plasmids are often lost at high frequency when cells are cultured in enriched, nonselective media. This curing does not occur as readily if the plasmids are cryptic (Kretschmer et al., 1975; So et al., 1978). To date, few phenotypes have been shown to be encoded by MAIS plasmids (Crawford et al., 1981b; Mizuguchi et al., 1981; Meissner and Falkinham, 1984), so that the vast majority of MAIS plasmids must currently be considered cryptic. The cryptic nature of most MAIS plasmids is underscored by the stability of MAIS plasmid profiles despite repeated growth in MGE broth. For example, the LR isolates provided to me by Dr. Crawford (Table 1; Chapter 2) have been continually maintained in enriched media for at least 6 years. And, despite repeated subculturing of these isolates by ourselves and by Crawford, neither group has observed any loss of plasmids. Thus, plasmid profiles fulfill the criterion of stability and reproducibility required for epidemiologic markers.

I screened for plasmids in 131 human and 226 environmental MAIS isolates; the largest and most comprehensive study of its kind to date. In the only other reported study, Crawford observed plasmids in 32 of 100 clinical isolates (J. T. Crawford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C 209, p. 306). Thus, ours was the first study which examined environmental isolates and compared them with human isolates from the same geographic areas. Like Crawford, I found that plasmids were common among human isolates regardless of their geographic origin from southern or eastern regions of the United States.

Because human infection by MAIS bacteria is encountered throughout the world (Tsukamura et al., 1974; Meissner and Anz, 1977; Tuffley and Holbeche, 1980), one wonders whether plasmids are common among human isolates from other areas, such as the western United States or even Japan, Australia, Europe, etc. Based on a report published by Mizuguchi (Mizuguchi et al., 1981), we know that at least some Japanese human isolates contain plasmids. It is conceivable that certain MAIS plasmids (or portions thereof) may be very widely distributed. This has been shown to be the case with certain R plasmids from Salmonella (Terakado et al., 1983; Casalino et al., 1984) and with chlorobenzoate-degradative plasmids from Pseudomonas (Chatterjee and Chakrabarty, 1983).

On the basis of my work, I have evidence that certain MAIS plasmids may be widely distributed. In Chapter 4 I showed that a human isolate deposited in the Trudeau Memorial Collection nearly 30 years ago and a recent Savannah River isolate both contained an identical 16.5 Md plasmid. Additionally, I found that 15 mercury resistant human and environmental MAIS isolates from diverse locations all contained a 115 Md plasmid which is likely identical to the 115 Md plasmid (pVT1) which encodes mercury resistance in M. scrofulaceum strain W262. The widespread distribution of pVT1 is highlighted by the fact that 115 Md plasmids were observed in mercury resistant MAIS such as the Canadian human isolate (TMC 1323) and the environmental isolate from Miami, Florida (W240). As discussed in Chapter 4, unequivocal proof of the identity of these 115 Md plasmids awaits development of techniques for isolation of a single mercury-resistance plasmid in sufficient amounts for Southern blot analysis.

One of the most significant achievements of this study was my discovery of the first selectable marker encoded by a MAIS plasmid (Meissner and Falkinham, 1984). The discovery of a selectable marker encoded by a MAIS plasmid will allow for future studies on important phenomena such as gene transmission. Although 5 years have passed since plasmids were detected in MAIS (Crawford and Bates, 1979), it is still not known whether plasmid transmission occurs between MAIS organisms.

I showed that the mercury resistance of M. scrofulaceum strain W262 resulted from the synthesis of a mercuric reductase which was encoded by a 115 Md plasmid. I identified 27 other mercury resistant MAIS isolates and I showed that they all volatilized mercury. Thus, the mechanism of mercury resistance in MAIS isolates is similar to the mechanism found in other aerobic bacterial genera (Robinson and Tuovinen, 1984). My studies with mercury resistant MAIS isolates have increased our understanding of the physiological ecology of these bacteria and, as discussed above, the work with mercury resistant isolates also led to important epidemiological findings.

Because of the heterogeneity of MAIS plasmid profiles, I rarely encountered two isolates which had identical profiles. The isolates which did have identical profiles were the following environmental isolates: 2s = 18s, 4s = 17s, 6s = 8s, 7s = 21s, W271 = W279, DE10 = DE17, W262 = CL25 = CL28. As can be seen, most of the isolates with identical profiles are aerosol isolates (refer to Table 1; Chapter 2 for the letter codes which denote aerosol isolates, etc.). Bacteria which share identical plasmid profiles are often considered to be clones (Orskov and Orskov, 1983; Jones, 1983; Riley et al., 1983). Therefore, the fact that we obtained environmental isolates with identical profiles allows

us to identify or describe the geographic range of mycobacterial clones. For example, the geographich range of the clonal population from which W262, CL25 and CL28 were drawn must be quite large. These 3 isolates (which can be considered to represent members of a single population) were obtained from samples collected at locations separated by over 100 miles (see Table 1; Chapter 2). Thus, plasmid profile analysis can serve as a simple method for testing the identity of separate bacterial isolates. However, one must recognize that identical plasmids can be shared by different bacterial species (Wallace et al., 1981; Perez et al., 1982) or even by different bacterial genera (Polak and Novick, 1982; Farrar, 1983). In this specific example, strains W262, CL25, and CL28 have the biochemical characteristics of M. scrofulaceum.

As a consequence of the heterogeneity of human and environmental MAIS plasmid profiles, it was not possible to correlate any particular plasmid profile definitively with a geographic region. However, as discussed in Chapter 4, I noted that plasmids in the 20 to 30 Md size range were most common among human and aerosol isolates from the Virginia/James River region. The similarity of the profiles of the human and aerosol isolates from this region supports our belief that MAIS in natural aerosols can contribute significantly to human colonization and disease (Parker, 1983). None of the plasmid profiles of other environmental isolates bore such stiking resemblance to human profiles. This result suggests that many environmental MAIS pose a smaller threat to public health relative to the threat posed by aerosolized MAIS.

As mentioned in Chapter 4, we question whether it is just by coincidence that aerosolized MAIS appear to also be human pathogens. For example, cell surface determinants that affect MAIS aerosolization could also enhance MAIS

pathogenicity. A possible mechanism for both phenomena could involve clumping. That is, clumping is known to enhance aerosolization concentration (Parker et al., 1983), and clumping would also be expected to increase pathogenicity because MAIS in clumps would be more likely to survive following phagocytosis by macrophages (Barksdale and Kim, 1977). Unusual clumping tendencies have in fact been observed in MGE broth cultures of aerosol isolates such as isolate 18s (unpublished observations). The exact nature of these cell surface determinants is being investigated (George and Falkinham, submitted) in aerosol experiments (Parker et al., 1983) and in pathogenicity experiments (Mapother and Songer, 1984; Thorel and David, 1984). If such determinants exist, then it is possible that plasmids may be involved in their expression. Such involvement has been shown to occur in other bacterial pathogens (Kopecko et al., 1980; Nandadasa et al., 1981; Heesemann et al., 1983). Our finding that aerosol and human MAIS isolates have similar plasmid profiles is consistent with our belief that plasmids may play a role in both aerosolization and pathogenicity.

Unfortunately, when the environmental MAIS isolates used in this study were being obtained, it was not known that aerosol isolates would become such interesting subjects for scrutinization. Therefore, only 16 aerosol isolates were available during our study. Increased emphasis is now being placed on the collection of large numbers of aerosol isolates from a variety of geographic locations. Plasmid profile analysis of increased numbers of aerosol isolates from a variety of different locations should help to clarify the epidemiologic relationship between aerosolized MAIS and MAIS from human infections. For example, if aerosol isolates from the Virginia/James River area continue to contain 20 to 30 Md plasmids, while aerosol isolates from other regions do not,

then plasmids of this size could serve as definitive epidemiologic markers for human disease in the Virginia/James River area.

Finally, I should emphasize that at the outset of this study absolutely nothing was known about the plasmid content of our collection of human and environmental MAIS isolates. During the course of this study I have obtained important information about the frequencies and functions of plasmids in these isolates. This work clearly indicates that plasmid analysis can strongly contribute to increased understanding of these interesting and important bacteria.

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