Plasmid analysis of *Mycobacterium avium-intracellulare* (MAI) isolated in the United Kingdom from patients with and without AIDS

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Summary. One hundred and forty-seven isolates (128 strains) of *Mycobacterium avium-intracellulare* (MAI) were screened by agarose gel electrophoresis for the presence of plasmids. Plasmids were characterised according to size and by Southern hybridisation analysis of intact and restriction endonuclease-digested DNA. Two cloned MAI plasmids, pLR7 and pLR20, were used as probes. There was no significant difference in the rate of plasmid carriage in MAI strains isolated from patients with the acquired immuno-deficiency syndrome (AIDS) and from non-AIDS patients in the UK, but a higher rate of plasmid carriage was observed in a panel of American strains from AIDS patients. Plasmids were grouped into two broad categories: small (mostly 14–30 kb) and large (> 150 kb). Southern blot analysis identified two distinct groups of small plasmids, the majority of which showed homology with pLR7. Plasmids from this group were significantly more common in strains of serotypes 4 and 8 which are particularly associated with AIDS.

Introduction

Organisms of the *Mycobacterium avium-intracellulare* (MAI) complex are the most common cause of systemic bacterial infection in patients with the acquired immunodeficiency syndrome (AIDS). Over 50% of AIDS patients may be infected with MAI at some stage of their disease.^{1–3}

The reasons for the close association between MAI and AIDS are not known. The pattern of disease suggests that infection occurs *via* the gastrointestinal tract rather than by the respiratory route.^{1–4} Infection is frequently associated with abdominal pain, diarrhoea and malabsorption. Large numbers of MAI organisms may be found in faeces and in biopsy tissue from the gastrointestinal tract, and the recovery of MAI from these specimens has been correlated with invasive disease.^{2–6}

The MAI complex comprises two biochemically closely-related species, *M. avium* and *M. intracellulare*, which may be distinguished by serological and genetic analyses. Good⁷ reported that over 60% of MAI isolates from patients with AIDS were either serotype 4 or 8. Both these serotypes are identified as *M. avium* by DNA homology studies.^{8, 9} Guthertz *et al.*¹⁰ used species-specific probes to analyse MAI isolates from patients with and without AIDS; 98% of strains from AIDS patients were *M. avium* compared with only 60% of strains from patients without AIDS. All the remaining strains were *M. intracellulare*. Therefore, it

appears that not all members of the MAI complex are equally capable of causing infection in patients with AIDS.

In 1986, Crawford and Bates¹¹ reported that all of 26 MAI isolates from patients with AIDS possessed plasmid DNA, and suggested that plasmids might have an important role in the pathogenesis of MAI infection in AIDS. Recent studies have associated plasmid carriage with virulence as determined by measurements of catalase activity,¹² mortality rates in beige mice and the release of oxygen metabolites by murine macrophages.^{13, 14}

We report a survey of plasmid carriage in MAI strains isolated in the UK from patients with and without AIDS. Plasmids were characterised according to size, by analysis of restriction digest patterns, and by Southern hybridisation with two cloned MAI plasmids as probes.

Materials and methods

Mycobacterial strains

MAI strains from patients with AIDS were either isolated at St Mary's Hospital or kindly provided by Dr P. A. Jenkins, Mycobacterium Reference Unit, Public Health Laboratory, Cardiff, Mrs M. Chadwick, The Brompton Hospital, London, and Mr M. Yates, Regional Centre for Tuberculosis, Dulwich, London, who also provided a panel of strains from patients who did not have AIDS. AIDS-related MAI isolates from the USA were obtained from Mr R. A. Ollar, St

Vincent's Hospital, New York, USA. Veterinary strains were donated by Dr E. Boughton, Central Veterinary Laboratory, Weybridge, and by the Regional Centre for Tuberculosis, Dulwich. MAI strains LR25 and LR113, which were used as standards in plasmid characterisation, were provided by Dr J. T. Crawford, The John L. McClellan Veterans' Hospital, Little Rock, AS, USA.

Serotyping of AIDS-related MAI isolates was performed at the Centers for Disease Control, Atlanta, GA, USA according to standard methods.¹⁵ Serological analysis was not available for strains isolated from patients without AIDS.

Plasmid screening

Screening of MAI for plasmid carriage was performed by a procedure modified from those of Crawford *et al.*¹⁶ and Kieser.¹⁷ Mycobacteria were grown for 14–16 days at 37°C in 10 ml of Middlebrook 7H9 Broth (Difco) containing oleic acid/albumin/dextrose/catalase (OADC) 10% v/v and glycerol 0.2% v/v. Cycloserine and ampicillin (Sigma) were added to final concentrations of 1.0 mg/ml and 0.1 mg/ml respectively. Bacteria were incubated for a further 20–22 h at 37°C, harvested by centrifugation (1000 *g*, 10 min) and resuspended in 500 µl of a freshly prepared solution containing lysozyme (Sigma) 2 mg/ml, 0.3 M sucrose, 25 mM Tris-HCl (pH 8.0) and 25 mM EDTA (pH 8.0). Suspensions were incubated for 3–24 h at 37°C. Cells were lysed by adding 250 µl of alkaline sodium dodecyl sulphate (SDS) 2% w/v in 0.3 M NaOH and incubation at 70°C for 20 min. Lysates were cooled, and extracted twice with 750 µl of phenol:chloroform (phenol 5 g; chloroform 5 ml). A 50-µl sample of clear aqueous phase was mixed with 10 µl of loading buffer (sucrose 60% w/v; 100 mM EDTA; bromophenol blue 0.25% w/v) and loaded on to a horizontal agarose 0.5% w/v gel for electrophoresis at 1.8 V/cm for 16 h in TAE buffer (40 mM Tris acetate, pH 7.9, 2 mM EDTA). Gels were stained for 30 min in ethidium bromide 0.5 µg/ml solution, destained for 1–2 h in distilled water, examined under ultra-violet illumination (302 nm) and photographed.

Plasmids were classified as small or large by virtue of their migration in agarose gels relative to two small plasmids of 18 kb and 28 kb and one large plasmid (> 150 kb) isolated from strain LR25.¹⁸

DNA was transferred over 20–24 h to nylon hybridisation membranes (Dupont GeneScreen Plus) by Southern blotting in alkaline conditions, with 0.4 M NaOH, 0.6 M NaCl as the transfer buffer. Membranes were neutralised by soaking for 15 min in 0.5 M Tris-HCl (pH 7.0), 1 M NaCl, dried and stored desiccated.

Probes

Two MAI plasmids, pLR7 and pLR20, that had been cloned into *Escherichia coli* were used as probes. No homology exists between these two plasmids. The

recombinant pLR7::pBR322 plasmid (pJC20) has been described elsewhere¹⁹ and was provided by Dr J. T. Crawford, The John L. McClellan Memorial Veterans' Hospital, Little Rock, AS, USA.

The second probe (pJC70) was derived from the plasmid pLR20 which was extracted from a serotype 4 MAI strain, isolated from an AIDS patient in the USA. The pLR20 plasmid was isolated and cloned (by T.J.H.) in collaboration with Dr Crawford at the Veterans' Hospital. Briefly, plasmid DNA was extracted from MAI strain LR541 (which was obtained by Dr Crawford from the Centers for Disease Control, Atlanta, GA, USA) as described above and further purified by the GeneClean procedure (Bio 101 Inc., La Jolla, CA, USA). DNA was digested with the restriction endonuclease *EcoRI* and ligated into the polycloning site of the *E. coli* vector pUC18²⁰ by standard methods. DNA from the ligation mixture was used to transform *E. coli* strain DH5a (amp^s lac⁻). Transformation and insertion of DNA were monitored by culture on medium containing ampicillin and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Bacteria carrying recombinant plasmids were unable to utilise the chromogenic substrate and formed white colonies. Minipreparations of plasmid DNA were performed and clones were characterised by analysis of restriction fragments and Southern hybridisation.

Probes were labelled with ³²P-deoxycytidine triphosphate by random hexanucleotide priming of DNA polymerase^{21, 22} by the Amersham Multiprime system (Amersham International, Little Chalfont, Berks).

Hybridisation

Prehybridisation, hybridisation and washing of membranes were all performed at 60°C. Membranes were prehybridised for 3–4 h in a mixture containing 6X SSC, 5X Denhardt's solution and SDS 0.5% w/v; 20X SSC is 3 M NaCl, 0.3 M sodium citrate; 10X Denhardt's solution is polyvinyl pyrrolidone (M_r 40 000) 0.02% w/v, ficol (M_r 400 000) 0.02% w/v and bovine serum albumin (fraction V) 0.02% w/v in water. Hybridisation was performed overnight in a mixture which differed from the prehybridisation fluid in that it also contained 0.01 M EDTA. Membranes were washed for 2 h in 0.1X SSC, SDS 0.1% w/v, with a change of washing solution every 30 min. Autoradiographs were exposed at -70°C to Amersham Hyperfilm with Rapid-X intensifying screens (Genetic Research Instrumentation, Dunmow, Essex).

Restriction digestion

DNA for restriction analysis was purified by anion exchange chromatography by the Qiagen DNA purification system (Diagen GmbH, Dusseldorf, Germany). Cells were grown with shaking at 37°C in 200 ml of Middlebrook 7H9 broth (containing OADC

10% v/v and glycerol 0.2% v/v) to mid-exponential phase. Cycloserine and ampicillin were added as previously described and incubation was continued for 20–22 h. Bacteria were harvested by centrifugation and resuspended in 4 ml of RNAase buffer (50 mM Tris-HCl, 10 mM EDTA, RNAase A, Sigma, 400 µg/ml). Cells were lysed at room temperature by addition of 4 ml of alkaline SDS (SDS 1% w/v; 0.2 M NaOH). Cell debris and SDS were precipitated by addition of 4 ml of potassium acetate (2.55 M, pH 4.8) and incubation on ice for 10 min. Lysates were centrifuged at 20 000 *g* for 30 min at 4°C and the clear supernate was loaded onto a Qiagen Tip-20 chromatography column that had been pre-equilibrated with buffer A (1 M NaCl, 50 mM morpholinopropanesulphonic acid (MOPS), ethanol 15% v/v, pH 7.0). DNA was allowed to bind to the chromatography resin by slow passage of the lysate through the column. The resin was washed with 6 ml of buffer B (1.2 M NaCl, 50 mM MOPS, ethanol 15% v/v, pH 7.0) to remove impurities, and the DNA was eluted with 2 ml of buffer C (1.2 M NaCl, 50 mM MOPS, ethanol 15% v/v, pH 8.0). DNA was precipitated with 0.8 volumes of isopropanol at room temperature, washed in ethanol 70% v/v and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Plasmids were compared by digestion with the restriction endonuclease *Bst*EII (Gibco BRL) for 2 h at 60°C in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM NaCl. DNA fragments were separated by electrophoresis through agarose 1% w/v gels at 3.6 V/cm for 4–5 h in TAE buffer. Gels were stained for 30 min in ethidium bromide 0.5 µg/ml solution, destained for 1 h in distilled water, examined under UV illumination and photographed. Analysis of restriction digests was complicated by the presence of more than one plasmid in many strains. DNA fragments were transferred to GeneScreen Plus hybridisation membranes as described above, and Southern hybridisation was performed with the two probes to identify fragments with homology to either pLR7 or pLR20.

Statistical analysis

Rates of plasmid carriage were compared by the χ^2 test.

Results

A total of 147 MAI isolates was examined for plasmid carriage. For the purpose of this study, isolates from the same patient which could not be distinguished by plasmid analysis were considered to represent the same strain. Eighty-eight isolates (71 strains) originated from 70 patients in the UK who had AIDS, nine isolates (nine strains) were from AIDS patients in New York, 36 isolates (34 strains) were from 34 non-AIDS patients in the UK, and 14 isolates (14 strains) were from animals in the UK.

Fig. 1. Agarose gel electrophoresis of crude plasmid DNA. Migration was from top to bottom. *M_s* indicated are those of plasmids isolated from strain LR25.¹⁸ lane f. Strain sources: a, b, UK non-AIDS patients; c, d, UK AIDS patient; e, US AIDS patients; g, veterinary. MAI isolates shown in lanes c and d were isolated from the blood and faeces of the same patient, and have indistinguishable plasmid banding patterns.

The electrophoretic screening procedure enabled the number and size of plasmids carried by each strain to be determined; a representative screening gel is shown in fig. 1. Plasmids were grouped into two broad size categories: small plasmids, the majority, between 14 and 30 kb, and large plasmids which were over 150 kb. The results obtained by screening MAI isolates in this manner are summarised in table I. There was no significant difference in the rate of plasmid carriage between MAI strains isolated in the UK from patients with and without AIDS. However, all the AIDS-associated strains from New York possessed at least one plasmid. Large and small plasmids were found in strains from both AIDS and non-AIDS patients. No large plasmids were isolated from the veterinary strains, although two strains did carry a small plasmid. No MAI strain was found to possess more than three plasmids.

Plasmids were further characterised by Southern hybridisation with two cloned MAI plasmids, pLR7 and pLR20, as probes. Most small plasmids from both AIDS- and non-AIDS-related MAI strains showed homology to pLR7 (table II), as did one of the small plasmids isolated from a veterinary strain. Hybridisation of small plasmids with the pLR20 probe was less

Table I. Carriage of plasmids by 147 MAI isolates from different sources

Origin of isolates	Number of isolates	Number of strains	Number (%) of plasmid-carrying strains	Frequency of plasmid-carrying patterns*				
				A	B	C	D	E
UK AIDS patients	88	71	34 (48)	13	4	5	4	8
USA AIDS patients	9	9	9 (100)	1	0	3	4	1
UK non-AIDS patients	36	34	12 (35)	6	0	2	2	2
Veterinary	14	14	2 (14)	2	0	0	0	0
Total	147	128	57 (45)	22	4	10	10	11

* A, single small (14–30 kb) plasmid; B, two small plasmids; C, one small + one large (> 150 kb) plasmid; D, two small + one large plasmid; E, single large plasmid.

Table II. Frequency and hybridisation patterns of small plasmids in MAI strains from different sources

Origin of isolates	Number of small plasmids	Number (%) hybridising with	
		pLR7	pLR20
UK AIDS patients	34	22 (65)	5 (15)
USA AIDS patients	12	8 (67)	3 (25)
UK non-AIDS patients	12	6 (50)	2 (17)
Veterinary	2	1 (50)	0 (0)
Total	60	37 (62)	10 (17)

common. The probes did not hybridise to either the large plasmids or chromosomal DNA. No small plasmids were found with homology to both probes.

Southern blot analysis of intact plasmids is a relatively crude method of determining the relationship between plasmids from different strains because hybridisation may be due to only small regions of homology. For this reason, we analysed restriction endonuclease digests of plasmid DNA by agarose-gel electrophoresis and Southern hybridisation. As has been reported previously,¹¹ there was considerable variation in size (15–30 kb) of pLR7-related plasmids (fig. 2a), and in the hybridisation patterns of digested

Fig. 2. Diversity in size and *BstEII* restriction patterns of pLR7-related plasmids. A. Plasmid DNA prepared with the Qiagen DNA purification system and subjected to electrophoresis in agarose 0.5%, TAE buffer at 1.8 V/cm for 16 h. Migration was from top to bottom. The fastest running bands correspond to supercoiled plasmid DNA. The position of the chromosomal DNA band is marked (X). Remaining bands correspond to partially denatured plasmid DNA. Strain sources: a, veterinary; b, unknown; c, d, UK non-AIDS patients; e–i, UK AIDS patients; j, strain LR113 (carrying the 15.3-kb pLR7 plasmid¹²). B. Autoradiograph of *BstEII* digests of plasmid DNA hybridised with recombinant pLR7. Strains are in the same lanes as in A. Linear size markers indicated are the *HindIII* fragments of phage λ .

Table III. Plasmid carriage, hybridisation patterns and isolation site of MAI isolates from different sources

Origin of isolate	Number of isolates*	Number (%) of isolates carrying			
		plasmids	plasmids hybridising with		large plasmids
			pLR7	pLR20	
<i>UK AIDS patients</i>					
Alimentary tract	28	13 (46)	7 (25)	2 (7)	7 (25)
Respiratory	25	12 (48)	7 (28)	2 (8)	7 (28)
Blood/bone marrow	17	8 (47)†	5 (29)	0 (0)	4 (24)
Other/unknown	9	6 (67)	6 (67)	1 (11)	2 (22)
<i>USA AIDS patients</i>					
Blood	9	9 (100)†	8 (89)	3 (33)	8 (89)
<i>UK non-AIDS patients</i>					
Respiratory	16	6 (38)	3 (19)	1 (6)	3 (19)
Other/unknown	18	6 (33)	3 (17)	1 (6)	3 (17)

* Identical isolates from the same site were counted once; identical isolates from different sites were counted separately.

† $p < 0.01$.

fragments (fig. 2b). In contrast, plasmids with homology to pLR20 were more consistent in size (c. 14–16 kb) and *Bst*EII-digest banding patterns (data not shown). There was some variation in the size of the large plasmids, although this could not be quantified accurately.

Plasmids with homology to pLR20 were always found in the presence of another small plasmid and were always the smallest plasmid present. Eight of 10 strains which possessed a plasmid related to pLR20 also carried a plasmid related to pLR7. Thirteen small plasmids were isolated (seven from AIDS-related and four from non-AIDS MAI strains isolated in the UK, one plasmid from an American AIDS MAI strain, and one MAI strain of veterinary origin) which were unrelated to either pLR7 or pLR20 by Southern blot analysis. These plasmids have yet to be characterised.

In patients with AIDS, similar rates of plasmid carriage were observed in MAI isolates from different parts of the body (table III). There was no significant

difference in the rate of plasmid carriage between MAI isolates from the respiratory tract in AIDS and non-AIDS patients. All the American AIDS-associated strains were isolated from blood and carried plasmids. In contrast, only 8 (47%) of 17 strains isolated from the blood or bone marrow of AIDS patients in the UK possessed plasmid DNA ($p < 0.01$).

It is possible that plasmid carriage and serotype are linked in the pathogenesis of MAI in AIDS. In this study (table IV), 14 (67%) of 21 serotype 4 or serotype 8 MAI isolates from UK AIDS patients carried plasmid DNA (in all cases including a pLR7-related plasmid). Amongst all other serotypes, 11 (44%) of 25 strains carried a plasmid, but only four of these strains possessed a plasmid which hybridised to pLR7. The difference in the carriage of pLR7-related plasmids between strains of serotype 4 or serotype 8 and strains of other serotypes was highly significant ($p < 0.005$).

Discussion

Our interest in MAI plasmids was prompted by the fact that they may help to explain the specific association between MAI colonisation and infection in late-stage HIV (human immunodeficiency virus) disease. MAI has been found in 17–28% of AIDS patients before death^{23, 24} and in over 50% of patients examined *post mortem*;^{1–3} yet this group of organisms is no more common in the environment than many other atypical mycobacteria. There is increasing evidence that the portal of entry may be the gastrointestinal tract.^{1–6} Work in progress at St Mary's Hospital suggests that individuals without HIV infection do not carry MAI in their faeces, and that MAI appears in the faeces at a late stage in the progression of HIV infection (unpublished observation).

Table IV. Plasmid carriage and serotype of AIDS-related MAI strains isolated in the UK

Serotype	Number of strains	Number of strains carrying	
		plasmids	plasmids hybridising with pLR7
1	4	2	2
4	15*	8	8
8	6	6	6
9	4	3	1
Not typable	4	2	0
Other	13	4	1
Total	46	25	18

* Includes two strains of mixed serotype; one of which carried a plasmid that hybridised to pLR7.

Analysis of AIDS-associated MAI strains suggests that they belong to a special sub-group of organisms defined by DNA fingerprinting,²⁵ serological type^{7, 26} and plasmid carriage.¹¹ The hypothesis we wish to test is that plasmids play a role in the establishment of MAI in AIDS patients.

Crawford and Bates¹¹ showed that all AIDS-associated MAI strains from a group of American patients carried plasmids. In contrast, in this study only 48% of AIDS-associated MAI isolates carried plasmids. This level was not significantly higher than the 35% plasmid carriage rate found in MAI isolates from clinical sources not associated with AIDS or HIV infection. All the AIDS-associated MAI strains of American origin that we examined possessed plasmids, but the sample of nine is too small to say whether this finding reinforces that of Crawford *et al.*, and that there is a real difference between AIDS-associated MAI strains from America and from the UK. The rate of plasmid carriage observed amongst clinical isolates of MAI not associated with AIDS was similar to that reported from Denmark by Jensen *et al.*,²⁷ but lower than the figures of 52% and 56% reported from Japan and the USA.^{28, 29}

The American strains we screened for plasmid carriage were all isolated from blood, but plasmid carriage could not be correlated with the site of isolation in strains from the UK. However, there was a strong association between plasmid carriage and serotypes 4 and 8 among the UK MAI strains. All the American strains analysed by Crawford and Bates¹¹ were serotype 4 or serotype 8. Unfortunately, the serotypes of our panel of American AIDS strains were not known. It would be interesting to determine whether American AIDS-associated MAI strains that were not of serotype 4 or serotype 8 had a lower rate of plasmid carriage. There is also a need to examine more environmental and veterinary strains.

We have used Southern hybridisation analysis to define two distinct groups of small plasmids in MAI strains. The most frequently isolated plasmids were those with homology to pLR7. Plasmids from this group were significantly more common in the serotypes most often associated with AIDS. However, carriage of a pLR7-related plasmid was not restricted to strains of these serotypes and possession of a large or small plasmid was not a pre-requisite for the ability to cause disease in patients with or without AIDS.

It is evident from the diversity in size and restriction-fragment patterns of pLR7-related plasmids that many carry only relatively short sequences with homology to pLR7. Therefore, these plasmids may encode a variety of different functions. There may be some co-ordination of function between different plasmids because pLR20-related plasmids were found only in the presence of another small plasmid. There is still

little information concerning the degree of relatedness amongst large plasmids isolated from different strains. This is primarily a result of their size and the difficulty in obtaining sufficient DNA with which to work. The large plasmids are present within the cell in low copy number and are easily damaged by the harsh conditions required to disrupt the mycobacterial cell wall and for the subsequent purification procedures. These problems may be overcome by cloning fragments of the large plasmids into suitable bacteriophage or cosmid vectors.

The existence of plasmids in MAI has been implicated in several characteristics;^{16, 28, 30, 31} of particular interest are the recent studies in which plasmids have been associated with the virulence of MAI.¹²⁻¹⁴ An important factor in the present context may be the ability to penetrate the gastrointestinal epithelium and survive within the gut-associated lymphoid tissues; mycobacterial cell-wall adhesins that react with receptors on the mucosal surface may be involved in this process.³²⁻³⁴ Plasmids are known to encode such properties in other organisms,³⁵⁻³⁷ and the importance of MAI plasmids in relation to these processes warrants further investigation.

The ability to distinguish MAI isolates from patients with and without AIDS by serological and genetic analysis^{7, 10, 25, 26} may simply reflect the characteristics of those strains present in the environment, but such observations may also serve as markers for differences in the pathology of MAI infection between these patient groups. We have established that plasmid profiles are useful epidemiological markers for the identification of repeat isolates of the same strain of MAI. In conjunction with serological typing and DNA fingerprinting, plasmid characterisation of clinical and environmental strains of MAI should enable potential sources of infection to be identified and establish the likely route of infection in patients with AIDS. Data from these typing systems would provide valuable insight into the importance of plasmid DNA in the ability of MAI to cause AIDS-associated disease.

Most studies on the functions of plasmids in MAI have been based on comparisons between ill-defined plasmid-free segregants produced by chemical mutagenesis.^{12, 13, 16, 30} However the recent development of suitable systems for the transfer and expression of foreign genes in mycobacteria³⁸⁻⁴¹ should help elucidate the roles played by plasmid DNA in the pathogenesis of this increasingly important group of organisms.

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