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Restriction Endonuclease Analysis of Members of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* Serocomplex

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Organisms belonging to the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* (MAIS) serocomplex were subjected to restriction endonuclease analysis (REA) with the enzymes *Bst*EII, *Pvu*II, and *Bcl*I. Substantial genetic heterogeneity was observed between members of an authenticated collection of the 31 serotypes. Serotypes 2 and 3 were indistinguishable, however, as were serotypes 5 and 10. No direct correlation could be made between restriction pattern and species identification. REA of serotype 2 and serotype 8 isolates from various geographic locations and animal origins showed that, within limits, the restriction pattern could be used as an index of serotype. Some isolates that were unable to be classified serologically exhibited restriction patterns identical to those of strains that were able to be classified by seroagglutination. The difficulty of interpreting much of the epidemiological data concerning MAIS organisms may be partially explained by the extent of heterogeneity observed by REA. These findings support the contention that the MAIS complex has a substantially greater degree of heterogeneity than has been revealed by traditional methods.

Strains of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* have been placed together as a distinct group and belong to what is known as the MAIS serocomplex (27, 38). Members of this group are widespread in nature, and despite their relatively low virulence for mammalian species they are among the most clinically significant of the so-called atypical mycobacteria (37). *M. avium* is characteristically a pathogen of birds; but organisms of all three MAIS species can produce disease in numerous other animal species, including deer, cattle, pigs, and humans (31). Recently, the involvement of MAIS organisms in disseminated infections has been of great interest since they have occurred in high frequency in patients with acquired immunodeficiency syndrome (2, 11, 13).

Although members of the MAIS complex can be differentiated from other mycobacterial species by biochemical tests, further subdivision has been difficult. Historically, the distinction between *M. avium and M. intracellulare* was based on their virulence in chickens (31). Numerical taxonomic analyses and other techniques have shown that the distinction between the two is very tenuous (20, 22, 25, 35, 36), whereas *M. scrofulaceum* can be distinguished from *M. avium* and *M. intracellulare* by biochemical and morphological characteristics (34). Schaefer (27) developed a serotyping system to subdivide the MAIS complex, and this has proved useful for ecological and epidemiological studies. This test is the principal means of differentiating the MAIS complex, which is now composed of 3 serotypes ascribed to *M. avium*, 25 to *M. intracellulare*, and 3 to *M. scrofulaceum* (38). Differentiation of *M. avium* and *M. intracellulare* from *M. scrofulaceum* has also been achieved by using immunodiffusion analysis of cell extracts (29), and this has shown some correlation with the Schaefer (27) seroagglutination system, as has the sensitin testing scheme described by Magnusson (17). There is also good correlation between the results of seroagglutination and the patterns of extracted bacterial lipids obtained by thin-layer chromatography (12). Adaptations of the serotyping and thin-layer chromatographic procedures have subsequently been used to formulate an authenticated collection of MAIS complex strains (33).

All of the described methods of MAIS strain differentiation have serious disadvantages when used for subspecies identification. Serotypes of many MAIS strains cannot be determined because they do not react with the available antiserum or because they autoagglutinate. This, in addition to the problems of cross-reactions of serotypes, makes the determination of a species very difficult. The numerical taxonomy analyses, the agglutination data, the immunodiffusion data, and the animal pathogenicity studies give support to the premise of Kubica and Silcox (14) that *M. avium* and *M. intracellulare* are variants of the same species, having a number of distinctive serotypes. There have thus arisen strong recommendations that *M. avium* and *M. intracellulare* be reduced to a synonym of *M. avium* (22).

In recent years, restriction endonuclease analysis (REA) of bacterial chromosomal DNA has been successfully used to assess the genetic relatedness among procaryotes (3, 19, 26), and these studies have been extended to the mycobacteria (5–7, 24, 28). The technique entails digestion with a restriction endonuclease followed by electrophoretic separation in an agarose gel. The restriction pattern becomes a fingerprint of the respective genome since cleavage occurs at specific sites, generally of 6 base pairs in length. In this study we investigated the potential of REA as a taxonomic aid to the classification of members of the MAIS serocomplex. Genetic similarities among reference strains of the 31 serotypes and among strains of an identical serotype were examined with the aim of developing a complementary method to serological classification of organisms belonging to the MAIS complex.

MATERIALS AND METHODS

Bacteria. Reference strains of each of the 31 serotypes of the MAIS serocomplex were obtained from D. J. Dawson (Laboratory of Microbiology and Pathology, Department of

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TABLE 1. Reference MAIS serotypes

Species and strain	Serotype
M. avium	
16909-2380[a]	1
P-194	2
19088	3
M. intracellulare	
10528-1079[a]	4
5688-46	5
Sweatman	6
AT214	7
S. J. Bull[a]	8
6450204[a]	9
Borne	10
041861424[a]	11
S76-93	12
S76-64	13
P-39[a]	14
Simpson[a]	15
Gamole	16
Cornell	17
S72-193	18
Huntly	19
S74-77	20
2993[a]	21
S76-110	22
2350	23
2154	24
72-888[a]	25
1994	26
Harrison	27
6845	28
M. scrofulaceum	
P-29	41
Lunning[a]	42
Brooks[a]	43

[a] Belongs to the authenticated serotype collection from the National Jewish Hospital (33).

Health, Brisbane, Australia) and are listed in Table 1. The remaining strains examined were isolated from diagnostic samples submitted to the Central Animal Health Laboratory. These consisted of 20 serotype 2 isolates from a variety of animals (cattle [12 strains], pig [3 strains], chicken [2 strains], opposum [2 strains], and duck [1 strain]) and 7 serotype 8 isolates (cattle [2 strains], pig [2 strains], opossum [1 strain], and human [2 strains]). Six MAIS strains which autoagglutinated and two strains which were untypable were also examined. Isolates were selected to cover a wide geographical spread within New Zealand. Strains were identified by their growth, colony characteristics, and biochemical tests (34).

DNA isolation. Bacteria were cultured in 100 ml of Tween-albumin broth (34) for 3 to 5 weeks. Cells were heat-killed at 70°C for 15 min and harvested by centrifugation at 5,000 × *g* at 4°C for 15 min. They were then washed once in 0.15 M phosphate-buffered saline (0.14 M NaCl, 4 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.3) and were either processed immediately or frozen at −20°C until required.

Mycobacterial DNA was extracted by the gentle lysis procedure described by Collins and de Lisle (5) and was purified by repeated dialysis in 10 mM Tris–1 mM EDTA (pH 8) buffer. DNA yield was determined by the fluorometric method described by Le Pecq and Paoletti (15), and purity was estimated by measuring the UV ratio at A_{260} and A_{280}.

REA. Restriction endonucleases were purchased from New England BioLabs, Inc., Beverly, Mass., with the exception of *Sma*I, which was obtained from Sigma Chemical Co., St. Louis, Mo. DNA (4 to 6 μg) was digested to completion with 40 to 60 units of each of the following enzymes under conditions specified by the manufacturers: *Apa*I, *Bcl*I, *Bst*EII, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Not*I, *Pvu*II, *Sfi*I, and *Sma*I.

Slab gel electrophoresis was performed with 1% agarose (ultrapure DNA grade; Bio-Rad Laboratories, Richmond, Calif.) in 440-mm-long casts run for 24 h at 130 V in recirculated Tris-acetate buffer (40 mM Tris, 5 mM sodium acetate, 1 mM sodium EDTA [pH 7.8]). Gels were stained for 1 h in ethidium bromide (0.5 μg/ml), placed on a UV transilluminator (Ultraviolet Products, San Gabriel, Calif.), and photographed (Tri-X film; Eastman Kodak Co., Rochester, N.Y.) by using a 120-format plate camera fitted with a gelatin filter (23A; Wratten).

Preparation of antisera. Bacterial suspensions (in phenolized phosphate-buffered saline) were heated for 15 min at 70°C. Rabbits were hyperimmunized by intravenous injection of fractions into the ear veins twice weekly for 3 weeks. Antisera were collected and titrated as described by Schaefer (27).

Seroagglutination. Serotyping of isolates was carried out by G. Meissner (Institut für Experimentelle Biologie und Medizin, Forschungsinstitut Borstel, Borstel, Federal Republic of Germany). The serotypes of some isolates were confirmed in this laboratory by the seroagglutination method described by Schaefer (27). Briefly, cells were grown on 7H10 agar for 2 to 3 weeks, harvested into phenolized phosphate-buffered saline, and standardized spectrophotometrically to an optical density at 525 nm of 0.4. Appropriate dilutions of antiserum and cell suspension were mixed and incubated at 37°C. Agglutination was read at 3 and 18 h.

RESULTS

DNA yields varied from 12 to 360 μg/g (wet weight) of cells, with wide fluctuations between different preparations of the same strain. The purity of the DNA extracts was usually high, with a mean and standard deviation of the A_{260}:A_{280} absorbance ratio of 1.8 ± 0.10. DNA from *M. scrofulaceum* serotypes 41 to 43 proved the most difficult to extract and gave the lowest yields. The restriction digests of DNA from these three serotypes produced poor fragment patterns which were difficult to analyze. The reasons for this are unclear.

In preliminary studies, DNA from several strains was digested with 10 restriction endonucleases. Since most of these enzymes were either unable to completely cleave the DNA or did not produce effectively resolved restriction patterns, further REAs of the MAIS serotypes were pursued with only three enzymes: *Bst*EII, *Pvu*II, and *Bcl*I. The reproducibility of the method was confirmed by repeating the REA on a selection of recultured strains.

The REA of the MAIS serocomplex was characterized by the wide extent of pattern diversity. For this reason the restriction patterns of all 31 reference serotypes are not shown, and only a representative selection is presented in Fig. 1 and 2 to illustrate the diversity. With each enzyme used, serotypes 2 and 3 were indistinguishable from each other, as were serotypes 5 and 10. The restriction patterns of all other serotypes, including those not shown in Fig. 1 and 2, were substantially different from each other.

REA was performed on DNA extracted from the serotype 2 and serotype 8 isolates from Wallaceville, New Zealand,

and the patterns were compared with those of reference serotypes 2 and 8 (Fig. 3). *Bst*EII digests of DNA from serotype 2 strains are shown in Fig. 3, lanes 1 to 4. *Bst*EII digests of DNA from serotype 8 strains are shown in Fig. 3, lanes 5 to 7. A total of 18 (90%) of the serotype 2 isolates possessed identical REA patterns. This pattern is shown in Fig. 3, lane 2 (pattern 2B). The REA pattern of the serotype 2 reference strain (Fig. 3, lane 1; pattern 2A) is almost identical to pattern 2B in that it possesses only one extra fragment line. Unique patterns exist for the two remaining serotype 2 isolates (Fig. 3, lanes 3 and 4; patterns 2C and 2D, respectively). Pattern 2C showed some resemblance to the major pattern, but little resemblance was shown by pattern 2D.

A similar result was found for the REA of serotype 8 strains, none of which were identical to the reference strain (Fig. 3, lane 5; pattern 8A). Three of the strains were indistinguishable (lane 6; pattern 8B) and showed some resemblance to pattern 8A. The four remaining strains were also indistinguishable (Fig. 3, lane 7; pattern 8C) but showed less resemblance to pattern 8A than those with pattern 8B.

DNA yields from the autoagglutinating and untypable strains were generally low (12 to 45 μg/g [wet weight] of cells), but DNA was able to be cleaved by using both *Bst*EII and *Pvu*II (Fig. 3, lanes 8 to 10). All six strains that autoagglutinated were found to produce identical restriction patterns (Fig. 3, lanes 8 and 9). This corresponded to a serotype 2B pattern. One of the untypable strains (Fig. 3, lane 10) also produced a pattern identical to that of serotype 2B, while the other possessed a unique pattern (data not shown) that did not correspond to any of the patterns of the reference serotype strains.

Uncut DNA from all strains was run on 0.7% agarose gels to test for the presence of plasmids. No plasmids were detected.

DISCUSSION

Yields of DNA from MAIS isolates were generally higher than those obtained from other slow-growing mycobacteria in this laboratory when similar methods were used (5–7). With the exception of the *M. scrofulaceum* extracts, the DNA was sufficiently pure to give clear, sharp restriction patterns. The comparison between patterns was easiest in the higher-molecular-size regions where fragment resolution was greatest.

Two pairs of reference serotypes possessed identical REA patterns (serotypes 2 and 3 and serotypes 5 and 10). There was a moderate degree of similarity between the lower-molecular-size regions of these four serotypes and serotype 1. In general, however, the most notable feature of the REA of MAIS serotypes was the high degree of diversity among them. Although some fragment lines were common to many serotypes, it was difficult to compare similarities because of this genetic diversity. Strains of the MAIS complex were not grouped into their respective species by REA, indicating that there is a large amount of genetic difference between most serotypes both between and within species. This is in marked contrast to the results obtained in similar studies on the tuberculosis complex (5, 6) and *M. paratuberculosis* (7), in which strains of the same species showed a high degree of pattern similarity. The extent of genetic heterogeneity between MAIS serotypes contrasts with the high degree of

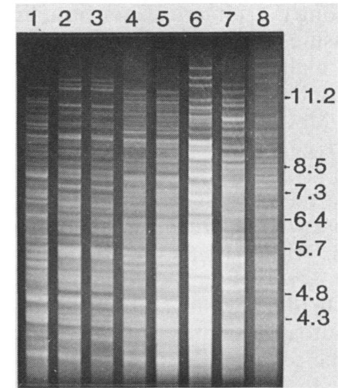

FIG. 2. Cleavage patterns produced by *Pvu*II digestion of DNA from the following: lanes 1 through 3, *M. avium* reference serotypes 1, 2, and 3, respectively; lanes 4 through 6, *M. intracellulare* reference serotypes 5, 10, and 15, respectively; lanes 7 and 8, *M. scrofulaceum* reference serotypes 41 and 42, respectively. Molecular size markers are expressed to the right of the gels in kilobase pairs.

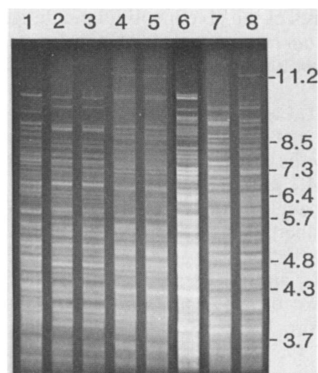

FIG. 1. Cleavage patterns produced by *Bst*EII digestion of DNA from the following: lanes 1 through 3, *M. avium* reference serotypes 1, 2, and 3, respectively; lanes 4 through 6, *M. intracellulare* reference serotypes 5, 10, and 15, respectively; lanes 7 and 8, *M. scrofulaceum* reference serotypes 41 and 42, respectively. Molecular size markers are expressed to the right of the gels in kilobase pairs.

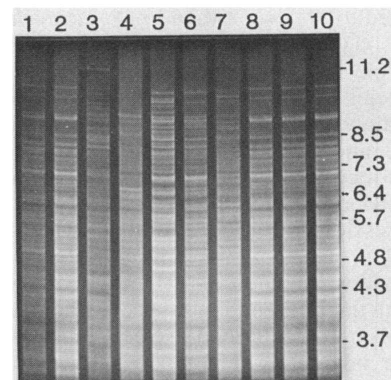

FIG. 3. Cleavage patterns produced by *Bst*EII digestion of DNA from the following: lane 1, *M. avium* reference serotype 2 (pattern 2A); lanes 2 through 4, serotype 2 isolates (patterns 2B through 2D, respectively); lane 5, *M. intracellulare* reference serotype 8 (pattern 8A); lanes 6 and 7, serotype 8 isolates (patterns 8B and 8C, respectively); lanes 8 and 9, autoagglutinating isolates; lane 10, untypable isolate. Molecular size markers are expressed to the right of the gels in kilobase pairs.

consistency among the numerical, sensitin, lipid pattern, and serological classification schemes (30). However, it is supported by the high degree of variability in guanine and cytosine ratios of MAIS serotypes, a finding which led Crowther and McCarthy (10) to suggest that the MAIS complex is more genetically diverse than indicated by traditional methods.

In contrast to the difference among serotypes, good correlation was observed between restriction patterns within a serotype. Thus, within limits, restriction pattern may be used to define serotype in a fashion similar to that of mycobacterial lipid analysis (12). The ability to classify isolates that autoagglutinate, with those of a particular serotype, may be particularly important since many clinical isolates rapidly lose their smooth colony morphology on repeated subculture.

REA may also be used to indicate the existence of yet unrecognized serotypes. This is important because up to 40% of clinical isolates may be untypable (16).

Epidemiological studies of MAIS organisms have often indicated a relationship between serotype and virulence (16). Serotypes 1, 2, and 8, for example, are usually the most common cause of MAIS infection in domestic animals, with serotype 8 being predominant in swine (16). It was not possible to relate virulence to any common feature of the restriction patterns. Although the REA patterns for the reference strains of *M. avium* serotypes 2 and 3 are identical, serotype 2 isolates have been isolated from animals up to 10 times more frequently than serotype 3 (16). Thus, minor variations in the genome may be very important in determining the virulence and geographical distribution of members of the MAIS group.

The designation of agglutinating serotypes 1 to 3 as *M. avium* serotypes, serotypes 4 to 28 as *M. intracellulare* serotypes, and serotypes 41 to 43 as *M. scrofulaceum* serotypes has been sustained more on a traditional basis rather than by a scientific rationale and succeeds as the most convenient classification scheme to date. Many researchers have attempted to classify members of the MAIS complex into subgroups (4, 12, 14, 17, 27, 29, 31, 33, 35). Meissner and Anz (21) reassessed the distribution of agglutinating serotypes between *M. avium* and *M. intracellulare*. They concluded, on the basis of virulence, that serotypes 4 to 6 and 8 to 10 consist of an intermediate group that is more closely related to *M. avium* than to *M. intracellulare*. Baess and Magnusson (1) provided the first conclusive evidence for the separation of *M. avium* and *M. intracellulare* into separate species by using DNA homology studies, and these homology data were in close agreement with the assessment of Meissner and Anz (21). Furthermore, the T-catalase studies described by Wayne and Diaz (35) also confirmed a similar redistribution of serotypes and led to the first quantitative expression of the genetic divergence seen in these organisms.

REA provides an additional method of examining the extent of both the inter- and intraspecific variation within the MAIS complex. It has limited use as a quantitative tool but is a suitable taxonomic adjunct to seroagglutination. The advantage of REA in epidemiology is its ability to identify small differences between isolates, even when this cannot be achieved by other methods. Thus, it can more reliably determine whether two or more different isolates have a common source.

Information regarding gene exchange in the mycobacteria is rudimentary, although a review (18) has revealed that many variations are caused by physicochemical agents, mycobacteriophages, and plasmids. In recent years the presence of plasmids in members of the MAIS complex has been documented and used in epidemiological investigations (9, 23). Such analysis has revealed an extreme heterogeneity of plasmids within clinical and environmental isolates. No two isolates were found to possess identical plasmid profiles. Significant disparity was also observed between the incidence of plasmids in clinical and environmental isolates. Phage typing of the MAIS complex has shown a relatively high incidence of lysogeny (32) and a wide distribution of phage lysis patterns (8). This indicates that the potential for extensive genetic recombination between MAIS organisms is present. The heterogenous nature of the REA shown here may be partially explained by such interactions.

Recently, interest in the MAIS complex has been heightened because of the increasing prevalence of disease among victims of the acquired immunodeficiency syndrome (2, 11, 13). In one major study, disseminated MAIS complex infections were associated with 55% of deaths (13). In addition, 77% of MAIS organisms were classified as serotype 4 and 85% possessed an intense yellow pigment. The reasons for this are unknown. Serotype 4 has become the most commonly isolated serotype among human MAIS isolates within the past decade. More extensive basic research into the molecular genetics of MAIS organisms and other mycobacteria is needed to help solve these problems.

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