

Analysis of Deoxyribonucleic Acids from Armadillo-Derived Mycobacteria

M. DE KESEL,¹ M. COENE,¹ F. PORTAELS,² AND C. COCITO^{1*}

Microbiology and Genetics Unit, ICP, University of Louvain, Medical School, Brussels 1200,¹ and Microbiology Unit, Institute of Tropical Medicine, Antwerp 2000,² Belgium

Armadillos are used for propagation of *Mycobacterium leprae* and are hosts of mycobacteria of the ADM (armadillo-derived mycobacteria) group. The deoxyribonucleic acids (DNAs) of isolates of the five phenetic subgroups of the ADM were analyzed and compared with the genomes of related bacteria. The guanine-plus-cytosine (G+C) contents of the DNAs were 62.2 to 67.1 mol% for different ADM strains, 56 mol% for *M. leprae* and LDC (leprosy-derived corynebacteria), and 62 to 71 mol% for reference mycobacteria. Restriction analysis showed neither adenine methylation in the GATC site (a specific trait of LDC strains) nor cytosine methylation in the CCGG and GGCC sites. Hybridization higher than 80% was obtained with DNA from isolates within the same ADM subgroups, whereas 17 to 50% hybridizations were observed with organisms from different ADM subgroups. Genomes of ADM strains and reference mycobacteria were 15 to 40% homologous, except for subgroups IV and V, whose DNAs were 54 to 62% homologous with *Mycobacterium lepraemurium* DNA. Little or no homology between *M. leprae* and ADM genomes was found. We concluded that single ADM subgroups can be considered as distinct species within the genus *Mycobacterium*; they are genetically unrelated to the other leprosy-associated bacteria.

The inability to propagate *Mycobacterium leprae* axenically has been a major obstacle to research work on leprosy. So far, only mice and armadillos have been used as sources of this microorganism for scientific investigation. Injection of *M. leprae* into a mouse footpad is followed by a limited local proliferation of this bacterium, which reaches a steady state in about 6 months (33). Subcutaneous or intravenous inoculation of leproma homogenates into nine-banded armadillos produces a lethal systemic disease; the animal dies in about 2 years and shows a dissemination of mycobacteria in the skin as well as in the parenchymal organs (25). Infected armadillo tissues are the starting material from which mycobacterial preparations for experimental and clinical work are obtained.

Recent studies have revealed, however, that wild (34) and captive armadillos (27, 29, 31) are the natural hosts of several mycobacterial species, including a group named ADM (armadillo-derived mycobacteria). Different ADM isolates have been classified into five subgroups (I to V) according to their nutritional requirements and mycolic acid compositions (Table 1) (32).

Moreover, the presence of a unique group of microorganisms, which were formerly called diphtheroids because of their morphological resemblance to the diphtheria agent, has been recognized in human leprosy tissues (see references 8 and 9 for a review). These gram-positive bacteria, which apparently are absent from the skin of healthy subjects, were subsequently identified as true corynebacteria (12, 17, 20, 21) and accordingly renamed LDC (leprosy-derived corynebacteria). The name "*Corynebacterium tuberculostearicum*" recently has been proposed on the basis of tuberculostearic acid production (7).

A knowledge of the properties of leprosy-associated organisms is essential, since preparations of armadillo-grown

M. leprae might contain these three types of bacteria (*M. leprae*, ADM, and LDC) in various proportions.

The aim of the present investigation was to analyze the deoxyribonucleic acids (DNAs) of a series of ADM isolates belonging to the five ADM subgroups. This work is based on the use of two original techniques specially developed for analysis of rare DNA species. A procedure for determination of base composition of nanogram quantities of DNA has been reported recently (10); DNA-DNA hybridization in the liquid phase is described below. In addition, we used restriction endonucleases to identify group-specific restriction patterns and thus distinguish among leprosy-associated microorganisms. The present work has taxonomic and clinical implications and might help to define the taxonomic position of leprosy-associated bacteria.

MATERIALS AND METHODS

Reference microorganisms. The following mycobacteria were used as reference strains: *M. tuberculosis*, *M. gordonae* (strains 10300 and 10305), *M. avium*, *M. lepraemurium*, and *M. scrofulaceum*. They were isolated and characterized by standard procedures (22) at the Institute of Tropical Medicine, Antwerp, Belgium. *Corynebacterium xerosis* (NCTC 9755), *Micrococcus lysodeikticus* (ATCC 4698), *Clostridium perfringens* (ATCC 3624), and *Escherichia coli* A19 (Max-Planck Institute, Berlin, Federal Republic of Germany) were from established culture collections. *M. lepraemurium* was multiplied in mice. Armadillo-grown *M. leprae*, which was purified by the Draper procedure, was provided by the World Health Organization.

ADM strains. The following ADM strains were used: 8483, 10546, 8480, and 8668 of subgroup I; 8507, 8489, 10119, and 8251 from subgroup II; 8637 and 9091 from subgroup III; 8837 from subgroup IV; and 8968 from subgroup V. These strains were isolated by F. Portaels (Institute of Tropical Medicine) from armadillo livers, according to a previously reported protocol (29). These organisms were multiplied in

* Corresponding author.

TABLE 1. Phenetic classification of ADM strains

Trait	Presence (+) or absence (-) in ADM subgroup ^a :				
	I	II	III	IV	V
Mycolic acid type ^b :					
α -Mycolates	+	+	-	+	+
methoxy-Mycolates	+	+	-	-	-
keto-Mycolates	+	+	+	+	+
ω -Mycolates	+	+	+	+	+
Tween 80 hydrolysis	-	+	-	-	+
Growth on glucose (sole carbon source)	+	-	-	+	-
Growth on Ogawa medium	+	+	-	+	+
Tolerance of 5% NaCl in growth medium	-	-	+	-	-

^a Data from reference 32.

^b See reference 26.

Ogawa medium at pH 6 and 37°C. Assignment of different ADM strains to given subgroups was done on the basis of mycolic acid composition and growth requirements (Table 1) as previously described in detail (28-32; F. Portaels, L. Larsson, J. Jimenez, and C. Cierkins, manuscript in preparation).

Purification of mycobacterial DNA. Suspensions of bacteria (10 mg) in 0.5 ml of 100 mM NaCl-1 mM ethylenediaminetetraacetate (EDTA)-50 mM tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.8) were incubated sequentially with 25 μ l of lysozyme (20 mg/ml, 14 h, 50°C), 25 μ l of pronase (20 mg/ml, 1 h, 37°C), and 25 μ l of sodium dodecyl sulfate (20%, 1 h, 37°C). The mixtures were extracted with chloroform-isoamyl alcohol (24:1, vol/vol), water-saturated phenol, and ether. After incubation with 5 μ l of ribonuclease (2 mg/ml, 1 h, 37°C), DNA solutions were purified by exclusion chromatography (Sephadex G50 columns equilibrated with 4.8 mM sodium phosphate, pH 7.8). Void volume fractions were purified on hydroxyapatite columns, which were washed first with 8 M urea-0.1 M sodium phosphate buffer (pH 7.8) containing 1% sodium dodecyl sulfate and then with 4.8 mM sodium phosphate (pH 7.8), and eluted with 480 mM sodium phosphate (pH 7.8) (10).

Double-labeling procedure for determination of base composition of DNA. The reaction mixture contained 7.5 mM MgCl₂, 0.75 mM dithiothreitol, 30 mM Tris hydrochloride buffer (pH 7.4), 5.25 μ M each deoxynucleoside triphosphate (dGTP, dTTP, and dATP), 5.25 μ M [5-³H]dCTP (28.5 Ci/mmol; New England Nuclear Corp., Boston, Mass.), 0.004 μ M [α -³²P]dATP (3,200 Ci/mmol, New England Nuclear Corp.), and 50 U of DNA polymerase I (type 104485 [Boehringer GmbH, Mannheim, Federal Republic of Germany], an endonuclease-containing preparation) per ml. After addition of DNA (0.5 μ g/10 μ l of reaction mixture) and incubation for 25 min at 20°C, the reaction was stopped by addition of ice-cold 10 mM EDTA-Tris hydrochloride buffer (pH 7.4) followed by 10% trichloroacetic acid containing 1% bovine serum albumin. Samples were filtered through glass fiber filters (MN85; Macherey-Nagel, Düren, Federal Republic of Germany), which were submitted to differential counting for ³H and ³²P in a scintillation spectrometer. The moles percent guanine-plus-cytosine (G+C) was obtained by the relation 1/mol% G+C = 0.01K³²P/³H + 0.01, the value of

K being determined for each experiment by using a few reference DNAs of known composition (10).

Restriction analysis of DNA. The following restriction endonucleases (Boehringer) were used: *Hpa*II, *Hind*III, *Hae*III, *Dpn*I, *Sau*3A, and *Eco*RI. *Mbo*I was from Pharmacia, Uppsala, Sweden. Some enzymes do not cleave methylated bases, others are active only on modified bases, and another group cleaves irrespective of base modification. DNA samples were incubated with single restriction nucleases according to the conditions established by the manufacturer and submitted to horizontal electrophoresis (250 V, 3 h) on a 0.5% agarose gel containing ethidium bromide, and plaques were photographed under ultraviolet light (260 nm) with a red filter (11).

DNA-DNA hybridization in liquid phase. The two DNA species to be annealed were submitted to 30-s sonication at 60 W (B12; Branson Sonic Power Co., Danbury, Conn.) in ice, yielding average segments of 10³ base pairs. One DNA species (0.02 μ g in 0.1 mM EDTA plus 10 mM Tris hydrochloride [pH 7.4]) was labeled by nick translation (10), using a precursor mixture of dGTP, dTTP, and dCTP (5.25 μ M each) plus 0.166 μ M [α -³²P]dATP (3200 Ci/mmol, 3.1 \times 10⁻³ μ mol/ml). The reaction was started by addition of polymerase I (15 U/ μ g of DNA) in 1 mM dithiothreitol-10 mM MgCl₂-46.7 mM Tris hydrochloride buffer (pH 7.5). The reaction was stopped by 10 min of incubation at 100°C. Unlabeled DNA (1.5 μ g/62 μ l, a 75-fold excess with respect to the labeled DNA species) was added, and the mixture was denatured for 10 min at 100°C. The final concentration of NaCl was increased to 0.3 M (14), and the mixture was incubated at the annealing temperature $t^\circ = T_m - 25^\circ\text{C}$, where T_m , the midpoint melting transition, was the average value of the two DNAs. The T_m value of a given DNA was obtained from the corresponding G+C value by the relation $T_m = 69.3 + 0.41(G+C) + 18.5 \log M$, where M is the ratio of the experimental concentration of NaCl (0.3 M in this case) to the reference concentration (0.195 M) (14). The length of the annealing reaction was made equal to twice the cot 1/2 value. The latter, which represents the product of the single-stranded DNA concentration (in moles of nucleotides per liter) by the time (in seconds) required to render half of the DNA double-stranded, was considered to be 1.03 for mycobacteria, according to earlier data (3-5). Hybridized DNA mixtures were incubated with S1 nuclease (30 U/ μ g of DNA) for 45 min at 37°C (2). Nuclease buffer contained 0.045 M NaCl, 0.033 M sodium acetate (pH 4.5), and 3 \times 10⁻⁴ M ZnSO₄. Hydrolysis was halted by addition of 1 ml ice-cold 10 mM EDTA-10 mM Tris hydrochloride buffer (pH 7.4) followed by addition of 2 ml of 10% trichloroacetic acid containing 2% bovine serum albumin. After 30 min in ice, mixtures were filtered on glass fiber filters and counted in a scintillation spectrometer. For each experiment, several controls were made to establish maximum (homologous DNA reassociation = 100% hybridization) and blank (reassociation with an unrelated DNA possessing a G+C value close to that of the unknown sample). In addition, kinetics of single- and double-stranded DNA hydrolysis by S1 nuclease, under our experimental conditions, were monitored for each annealing DNA. The graphs shown in Fig. 1, which compare the single-stranded DNA specificity of three concentrations of S1 nuclease, point to the chosen concentration of 30 enzyme units per μ g of DNA as the optimum one affording a 93% digestion of single-stranded DNA, with 5% cleavage of the double-stranded species. In the present work, blanks were obtained by hybridizing ADM DNA (62.2 to 67.1 mol% G+C according to Table 2) with DNA of *M. lysodeikticus*

(71.9 mol% G+C), and *M. leprae* DNA (56 mol% G+C) with DNA of *E. coli* (51.9 mol% G+C) (13).

RESULTS

Base compositions of DNAs from ADM strains. The G+C of DNAs of 11 strains belonging to the five ADM subgroups was determined by the double-labeling procedure described above. As a control, the buoyant densities of selected samples were also measured by ultracentrifugation in CsCl gradients.

Different ADM strains had G+C values of 62.2 to 67.1 mol%. A 5-U range was also found in ADM subgroup I, whereas groups II (2 U) and III (0.2 U) appeared more homogeneous. These data point to the genetic heterogeneity of ADM strains, which have base compositions overlapping the entire range of mycobacterial DNA (62 to 71 mol% G+C).

Restriction analysis of ADM strains. Restriction analysis can recognize specific base patterns and identify unique sequences carrying modified bases. Accordingly, we sought two restriction patterns for the methylation of adenine and cytosine in ADM DNAs.

The first system included the following endonucleases: *Mbo*I, to which DNA carrying a methylated adenine in the GATC recognition sequence is insensitive; *Dpn*I, which cleaves only the methylated sequence; and *Sau*3A, which recognizes GATC irrespective of the presence of methylated adenine. DNA of ADM strain 8968 was cleaved by *Mbo*I and *Sau*3AI (Fig. 2, lanes d and f), whereas the electrophoretic pattern with *Dpn*I was similar to that of the control (Fig. 2, lanes c and e). The other ADM strains yielded similar restriction patterns. Therefore, adenine within the restriction sequence GATC of ADM DNAs is not methylated.

The second system included the following endonucleases: *Hae*III, which recognizes the GGCC sequence unless the central cytosine is methylated; and *Hpa*II, to which the CCGG sequence carrying a methylated cytosine is insensitive. Cleavage of ADM DNA by *Hpa*II (Fig. 2, lane g) excludes the presence of methylated cytosine in the CCGG sequence, and that by *Hae*II (lane j) points to a lack of methylation of the internal cytosine in the GGCC recognition site.

DNA homology of ADM and reference mycobacteria. Homologous hybridization experiments were performed to as-

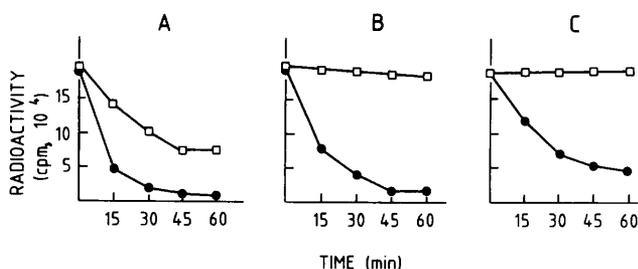


FIG. 1. Kinetics of DNA hydrolysis by S1 nuclease. The DNA of *M. gordonae* 10300 (19 µg/ml) was labeled by nick translation, and the reaction was stopped by 10 min of heating at 100°C. The NaCl concentration was raised to 0.3 M, and a sample was divided into two aliquots: one was incubated at 73°C for 72 h (reassociation close to 100%), while the other was stored at -20°C. Each sample was split into three parts which were incubated at 37°C with 100 (a), 30 (b), or 20 (c) U of S1 nuclease per µg of DNA. At the indicated times, aliquots were withdrawn from every sample for counting trichloroacetic acid-insoluble radioactivity. Symbols: □, double-stranded DNA; ●, single-stranded DNA.

TABLE 2. Base composition of DNAs from ADM strains and reference bacteria

Microorganism	DNA base composition (mol% G+C)	
	Experimental ^a	Previously reported (reference)
ADM I		
8480	66.8 ± 0.59	
8668	63.7 ± 0.39	
8483	62.2 ± 0.25	
10546	65.6 ± 1.06	
ADM II		
8507	67.1 ± 0.25	
8489	65.9 ± 0.87	
10119	64.7 ± 0.77	
ADM III		
8637	64.4 ± 0.52	
9091	64.2 ± 0.45	
ADM IV 8837	63.5 ± 0.87	
ADM V 8968	66.9 ± 1.05	
<i>M. tuberculosis</i>	66.5 ± 0.74	66.3 (4)
<i>M. gordonae</i> 10300	62.1 ± 1.51	63.3 (19)
<i>M. gordonae</i> 10305	63.9 ± 0.99	
<i>M. avium</i>	68.4 ± 0.4	70.3 (4)
<i>M. leprae</i>		54.8 (Hottat et al., in press) 55.8 (19)
<i>M. lepraemurium</i>		65.5 (19)
<i>M. scrofulaceum</i>		70.2 (4)
<i>C. xerosis</i>	59.8 ± 0.86	58.8 (13)
LDC		56.0 (Hottat et al., in press)

^a Determined by the double-labeling procedure (see the text); average of five independent determinations ± standard deviation.

sess the genetic homogeneity of ADM as a whole, and of its subgroups (which were established according to a series of phenotypic traits [Table 1]). Heterologous hybridization of the genomes of ADM strains and reference mycobacteria was done to establish the taxonomic position of the former organisms within genus *Mycobacterium*. DNA-DNA hybridization in the liquid phase (see above) was used for this purpose. Conditions for S1 nuclease-catalyzed hydrolysis of single-stranded DNA are of paramount importance (Fig. 1).

There were quite variable degrees of homology (15 to 90%) among the DNAs of members of the ADM in general (Table 3). Higher hybridization levels were found within ADM subgroups I and II (82 to 88%).

On the other hand, the hybridization of ADM DNAs with reference mycobacterial DNAs was relatively low (14 to 41%) (Table 3). An unexpectedly high homology (54 to 62%) between ADM subgroups IV and V on the one hand, and *M. lepraemurium* DNA on the other hand, was recorded.

Of particular interest is the very low hybridization level (1 to 12%) of *M. leprae* DNA with the genomes of different ADM strains. The former organism bore little homology (2 to

TABLE 3. DNA homology of ADM strains and reference bacteria

Source of unlabeled DNA	% hybridization ^a of labeled DNA from strain:				
	8480	8507	8837	8968	<i>M. leprae</i>
ADM I					
8480	100 ± 5.2	37.0 ± 4.6		15.5 ± 1.0	12.1 ± 8.0
8668	88.3 ± 5.9	45.4 ± 0.8			
ADM II					
8507	35.2 ± 2.5	100 ± 2.6		40.9 ± 0.6	3.1 ± 1.3
8251		82.5 ± 2.6			
ADM III					
8637	26.0 ± 3.1	24.3 ± 1.0		18.1 ± 2.6	10.7 ± 0.8
9091	22.3 ± 4.0	34.1 ± 2.8	19.8 ± 6.1		
ADM IV 8837	38.3 ± 4.6	16.9 ± 4.6	100 ± 4.6	24.0 ± 4.6	1.2 ± 0.4
ADM V 8968	18.6 ± 2.0	48.4 ± 9.6	28.5 ± 3.3	100 ± 1.4	5.5 ± 0.5
<i>M. lysodeikticus</i>	0 ± 0.8	0 ± 1.9	0 ± 1.6	0 ± 0.1	
<i>E. coli</i>					0 ± 0.6
<i>M. leprae</i>					100 ± 3.0
<i>M. avium</i>	20.3 ± 2.3	32.9 ± 4.9	14.5 ± 7.7	18.1 ± 1.6	3.3 ± 0.7
<i>M. gordonae</i> 10300	28.2 ± 1.9	30.4 ± 4.6		18.9 ± 4.8	
<i>M. scrofulaceum</i>	41.0 ± 2.4	37.4 ± 6.1	17.5 ± 0.5	17.0 ± 2.4	7.3 ± 1.1
<i>M. tuberculosis</i>	36.5 ± 5.3	39.7 ± 2.9	39.5 ± 0.5	41.5 ± 2.3	65.5 ± 10.3
<i>M. lepraemurium</i>	25.0 ± 3.9	25.5 ± 0.7	54.1 ± 3.0	61.5 ± 7.5	12.2 ± 9.2
<i>C. xerosis</i>	6.0 ± 0.5	7.3 ± 0.7	3.8 ± 0.4	10.1 ± 2.2	2.4 ± 0.4

^a Average of three independent determinations ± standard deviation.

12%) to the reference mycobacteria, except for *M. tuberculosis* (66%).

DISCUSSION

The present work was made feasible by the development of two microanalytical techniques for determination of the G+C content of DNA and DNA-DNA hybridization in liquid phase. The former procedure was the subject of a previous publication (10), whereas the latter is described above. Because ADM strains are fastidious, slow-growing bacteria, only a small amount of material is available for chemical analyses; application of the usual procedures (biophysical and chemical determination of DNA base content, and hybridization on solid supports) would not be practical. Both techniques are based on DNA polymerase-directed nick translation of the DNA, a very sensitive reaction requiring a highly purified template (10; Hottat et al., in press). Consequently, a procedure for purification of mycobacterial DNA, which is a difficult task, has been developed (10). All of these methods, on which the present work relied, are expected to help research projects on mycobacterial DNA.

Bacteria of the ADM group have been assigned to genus *Mycobacterium* on the basis of a series of phenetic traits (28–32; Portaels et al., in preparation). The G+C contents of DNAs from different ADM strains were in the range of 62.2 to 67.1 mol% (Table 2), which is almost as large a variation as that within the entire genus *Mycobacterium* (61 to 71 mol%) (6, 18, 24). It is clear, therefore, that this range largely

exceeds the species limits and encompasses that of a genus. This conclusion agrees with hybridization data (Table 3) showing 15 to 90% homology among the genomes of different ADM isolates, since two organisms displaying a homology degree equal to or higher than 15% belong to the same genus (24).

The homology level between two ADM strains belonging to different subgroups varied within a wide range (15 to 45%) (Table 3). On the contrary, the genomes of ADM strains within the same subgroup had higher percentages of hybridization and a smaller range of variation (82.5 to 88.3%). Although the latter conclusion relies on few determinations and is restricted to subgroups I and II, it can be provisionally proposed that each subgroup encompasses a species domain within the ADM and within the genus boundaries (24). If confirmed by further work on additional strains of the five ADM subgroups, this conclusion would support the present ADM classification, which was built on phenetic traits. Some DNA-DNA homologies between ADM have been reported previously (32). A disagreement between previous and present data concerns the genomes of ADM subgroups I and III, which were formerly found to be genetically indistinguishable, whereas in the present study they were clearly distinguished. The presence of impurities in DNA preparations, rather than the labeling and annealing procedures, might account for such discrepant results.

Three kinds of bacteria are associated with leprosy: *M. leprae*, ADM, and LDC. The taxonomic position of the former microorganism is uncertain because the G+C content

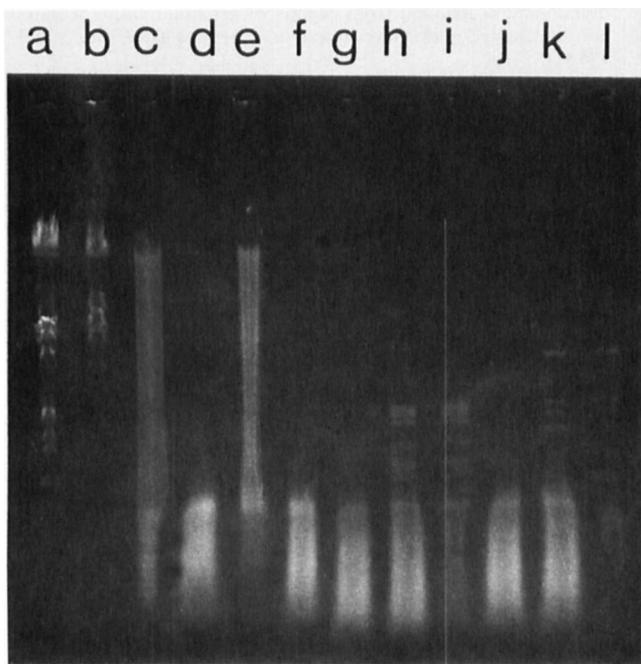


FIG. 2. Restriction patterns of ADM DNAs. DNAs from strain 8968 (ADM subgroup V) and bacteriophage λ (reference) were incubated with restriction endonucleases and electrophoresed. DNAs (lanes): strain 8968 (c to g and j), phage λ (a, b, i, and l), and strain 8968 plus phage λ (h and k). Endonucleases (lanes): none (c), *Mbo*I (d), *Dpn*I (e), *Sau*3AI (f), *Hpa*II (g to i), *Hae*III (j to l), *Hind*III plus *Eco*RI (a), and *Eco*RI (b).

of its genome (56 mol%) (19; Hottat et al., in press) does not match that of mycobacteria (61 to 71 mol%), and its peptidoglycan differs from that of all members of the gram-positive corynebacteria-mycobacteria-nocardia group (15, 16). LDC, on the other hand, are genetically (56 mol% G+C) (12; Hottat et al., in press) and phenetically (cell wall composition) (1, 17, 20, 21) related to corynebacteria, although strongly immunologically related to *M. leprae*. Recent work with restriction enzymes has revealed the presence of a unique marker in LDC DNAs: the $G_{m}TC$ sequence occurring in all LDC isolates but in no other member of the corynebacteria-mycobacteria-nocardia group (Hottat et al., in press). This sequence (Fig. 2) is absent from ADM and *M. leprae* DNAs. In addition, ADM strains share a low homology level with *M. leprae* (Table 4). Hybridization levels of *M. leprae* and LDC DNAs are also negligible (Antoine et al., unpublished data). The overall conclusion is that the genomes of the three groups of leprosy-associated bacteria can be easily distinguished by their G+C contents and restriction patterns and are genetically unrelated as judged by hybridization data.

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