Size and homology of the genomes of leprosy-derived corynebacteria, *Mycobacterium leprae*, and other corynebacteria and mycobacteria

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Summary. The genomes of *Mycobacterium leprae* and leprosy-derived corynebacteria (LDC), which have a similar base composition of guanine + cytosine 56 mol %, have been compared with those of reference bacteria of the CMN group (genera *Corynebacterium*, *Mycobacterium*, *Nocardia*). Genome sizes of three LDC strains were $(1.2\text{--}2.5) \times 10^6$ base pairs. DNA from four of seven LDC strains examined had homology levels >60%. Two other strains had a homology of 40% when compared with the CMN strains and one strain was distinctly different. The DNA from all seven LDC strains gave 0.3–18% hybridisation with that of *M. leprae*, 5–16% with reference corynebacteria, 5–12% with *M. bovis*, and 2–8% with *Nocardia caviae*. The small size of the LDC genome and its unrelatedness to those of *M. leprae* and organisms of the CMN group shows the uniqueness of LDC.

Introduction

Two types of micro-organisms have been found in leprosy lesions (Cocito and Delville, 1983, 1985). Those are *Mycobacterium leprae*, the causative agent of the disease, and diphtheroid organisms which have been isolated from the skin and blood of leprosy patients (Beaman *et al.*, 1974; Delville and Pichel, 1975). *M. leprae* can, at present, be cultivated only in nine-banded armadillos and in conventional or nude mice. The diphtheroids, which are not acid-fast, can be grown in pure culture and have recently been identified as corynebacteria by the presence of corynomycolic acid (Janczura *et al.*, 1981a; Gailly *et al.*, 1982) and a DNA base composition of guanosine + cytosine (G + C) 56 mol % (Danhaive *et al.*, 1982). These organisms have been designated “leprosy-derived corynebacteria” (LDC) (Cocito and Delville, 1983). Another kind of unusual bacteria has been isolated from armadillos in the wild and in captivity, whether or not they had been inoculated with leproma homogenates (Portaels *et al.*, 1985). These have recently been identified as mycobacteria by the presence of mycolic acid (Portaels *et al.*, 1986) and a DNA base composition of G + C 62–67 mol % (De Kesel *et al.*, 1987).

M. leprae has several unusual features. Its

peptidoglycan differs from that of bacteria of the genera *Corynebacterium*, *Mycobacterium*, *Nocardia* (CMN group), in that the L-lysine is replaced by glycine (Draper, 1976). In addition, its genome size is 1.3×10^9 base pairs (bp) (Imaeda *et al.*, 1982), or 2.2×10^9 bp (Clark-Curtiss *et al.*, 1985), and this is smaller than that of most bacteria. Moreover, the base composition of *M. leprae* DNA is G + C 56 mol % (Imaeda *et al.*, 1982) whereas that of other mycobacteria is G + C 62–70 mol % (Barksdale and Kim, 1977; Goodfellow and Wayne, 1982).

LDC share two diagnostic characteristics with conventional corynebacteria. Their mycolic acids are of the corynomycolic type (Gailly *et al.*, 1982) and their DNA base composition is G + C 56 mol % (Barksdale, 1970; Danhaive *et al.*, 1982). However, LDC have certain specific characteristics that differ from those of reference corynebacteria. Their peptidoglycan muramate is glycolylated (Janczura *et al.*, 1981b), tuberculostearic acid is present (Brown *et al.*, 1984), and the GATC sequences in their DNA contain N⁶-methyl-adenine (Hottat *et al.*, 1987). LDC are immunologically more closely related to *M. vaccae*, *M. nonchromogenicum*, *M. bovis* and other mycobacteria, than to *C. diphtheriae*, *C. hoffmannii*, *C. xerosis* and other corynebacteria (Ridell, 1977; Laub *et al.*, 1978; Gueur *et al.*, 1983; Abou-Zeid *et al.*, 1985).

The aim of the present work was to compare the genomes of leprosy-derived corynebacteria, *M. leprae* and reference bacteria of the CMN group.

Materials and methods

Micro-organisms

Leprosy-derived corynebacteria strains LDC 3 and 4 were originally isolated by J. Delville (University of Louvain, Brussels) from the blood of patients with borderline and tuberculoid cases of leprosy in Zaïre. LDC 8, 11, 15 and 24 were isolated by J. Delville and L. Barksdale (New York University, New York) from skin lesions of patients with lepromatous leprosy in Zaïre, Ethiopia, and the USA (table I). Some of these strains are deposited at the American Type Culture Collection (reference numbers in table I). LDC strain A5 and *M. leprae* were isolated by J. Delville from armadillos experimentally infected with material from lepromatous granulomas. Reference type strains were: *C. diphtheriae* PW8 from D. E. Minnikin (University of Newcastle upon Tyne), *C. xerosis* NCTC 9755, *M. bovis* BCG (Pasteur Institute, Paris), *N. caviae* ATCC 14629.

LDC strains and CMN organisms were grown in pure culture, in modified Dubos medium (Cocito and Delville, 1985) supplemented with 10% calf serum, under forced aeration in rotatory shakers. Armadillo-grown *M. leprae* (Kirchheimer and Storrs, 1971) was purified as reported by Draper (1976) and provided by the leprosy section of WHO.

DNA purification

Suspensions of bacteria (10 mg) in 0.5 ml of 100 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA), 50 mM Tris (hydroxy-methyl) aminomethane buffer (pH 7.8) were incubated sequentially with 25 µl of lysozyme (20 mg/ml, 14 h, 50°C), 25 µl of pronase (20 mg/ml, 1 h, 37°C) and 25 µl of sodium dodecyl sulphate (20%, 1 h, 37°C). The mixtures were extracted with chloroform:

isoamyl alcohol (24:1, v:v), water-saturated phenol and ether. After incubation with 5 µl of ribonuclease (2 mg/ml, 1 h, 37°C), DNA solutions were purified by exclusion gel 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 sodium dodecyl sulphate 1%, and then with 4.8 mM sodium phosphate (pH 7.8). Columns were eluted with 480 mM sodium phosphate (pH 7.8) (De Kesel *et al.*, 1987; Hottat *et al.*, 1987).

DNA hybridisation in liquid medium

The two DNA species to be annealed were sonicated for 30 s at 60 W (B12 sonifier; Branson Sonic Power Co., Danbury, CT, USA) in ice. DNA segments had an average size of 10^3 base pairs as determined by electrophoresis in agarose gels with *Hind* III-cleaved phage λ-DNA as reference. One DNA species (0.02 µg in 10 µl of 0.1 mM EDTA plus 10 mM Tris hydrochloride, pH 7.4) was labelled by nick translation (Coene and Cocito, 1985), with a precursor mixture of dGTP, dTTP, and dCTP (5.25 µM each) plus 0.166 µM (α - 32 P) dATP (3200 Ci/mmol, 3.1×10^{-3} µmol/ml). The reaction was started by addition of polymerase I (15 U/µg of DNA) in 1 mM dithiothreitol, 10 mM MgCl₂, 50 mM Tris hydrochloride buffer (pH 7.5). The reaction was stopped by immersing the reaction tube at 100°C for 10 min. The average specific activity of the probes was 5×10^7 dpm/µg of DNA. A 75-fold excess of unlabelled DNA (1.5 µg/62 µl) was added and the mixture was denatured by heating at 100°C for 10 min. The final concentration of NaCl was increased to 0.3 M, and the mixture was incubated at the annealing temperature $t^\circ = T_m^{\text{av}} - 25^\circ\text{C} = 71^\circ\text{C}$, where T_m is the midpoint hyperchromic

Table I. Origins of micro-organisms analysed

Strain designation	Origin			
	Tissue	Type of leprosy	Country	Strain no.
LDC 3 (D40)	blood	borderline leprosy	Zaire	ATCC 35517
LDC 4 (D43)	blood	tuberculoid leprosy	Zaire	ATCC 35518
LDC 8 (L3)	skin	lepromatous leprosy	Zaire	ATCC 35521
LDC 11 (0122)	skin	lepromatous leprosy	Zaire	—
LDC 15 (D86)	skin	lepromatous leprosy	Ethiopia	—
LDC 24 (2628)	skin	lepromatous leprosy	USA	ATCC 35693
<i>Mycobacterium leprae</i>	armadillo inoculated with lepromatous granuloma			
LDC-A5	armadillo inoculated with lepromatous granuloma			
<i>Escherichia coli</i> (strain A19)	(Max-Planck Institute, Berlin Germany)			
<i>Corynebacterium diphtheriae</i> (strain PW8)	(D. E. Minnikin, Newcastle-upon-Tyne)			
<i>Corynebacterium xerosis</i>	NCTC 9755			
<i>Mycobacterium bovis</i> (BCG)	(Pasteur Institute, Paris, France)			
<i>Nocardia caviae</i>	ATCC 14629			
	ATCC 14629			

transition at 260 nm of the DNA melting curve and T_m^{av} was the average of the T_m values of the two DNAs to be annealed (Baess and Bentzon, 1978; Baess, 1979). The T_m value of a given DNA was calculated from its G + C content according to the relation $T_m = 69.3 + 0.41 (G + C) + 18.5 \log M$, where M was the ratio of the NaCl concentrations of the unknown DNA (0.3 M in this case) and of reference DNA (that of *E. coli*, the T_m of which was determined in 0.195 M NaCl) (Dove and Davidson, 1962). The length of the annealing reaction was made equal to twice the Cot 1/2 value (Johnson, 1985) of the product of the single-stranded DNA concentration (moles of nucleotides/L) and the time in seconds required to render half of the input DNA double-stranded. DNA mixtures hybridised under stringent conditions were incubated with S1 nuclease (5 U/ μ g of DNA) for 30 min at 37°C (De Kesel *et al.*, 1987). Nuclease buffer contained 0.045 M NaCl, 0.033 M sodium acetate (pH 4.5), and 3×10^{-4} M $ZnSO_4$. Hydrolysis was stopped by adding 1 ml of ice-cold 10 mM EDTA, 10 mM Tris hydrochloride buffer (pH 7.4) followed by 2 ml of 10% trichloroacetic acid containing serum albumin 0.02%. After 30 min in ice, mixtures were filtered through glass fibre filters and counted in a scintillation counter. For each experiment, several controls were included. Homologous DNA reassociation, which varied between 60 and 90% of the input, was taken as 100% hybridisation. A blank sample was prepared with an unrelated DNA of G + C content close to that of the unknown sample, in this case *Escherichia coli* DNA. In addition, kinetics of single- and double-stranded DNA hydrolysis by S1 nuclease, under our experimental conditions, were monitored for each annealing DNA. The optimum concentration giving 89% digestion of single-stranded DNA and 9.8% cleavage of the double-stranded species was found to be 5 enzyme units of S1 endonuclease per μ g of DNA (De Kesel *et al.*, 1987).

Measurement of genome size

The size of labelled LDC DNA was measured at 71°C by analysis of renaturation kinetics (De Ley *et al.*, 1970; Baess and Mansa, 1978). The Cot 1/2 value of the product of the initial concentration of denatured DNA (moles of nucleotides/L) and the time (s) giving 50% re-annealing, was determined by graphic interpolation from the kinetic plots (Britten and Kohne, 1968; McFadden *et al.*, 1987). The technique used was similar to that outlined above, except that samples were periodically withdrawn from the reaction mixture for incubation with S1 nuclease. The enzyme action was stopped by addition of ice-cold trichloroacetic acid and, after filtration, the radioactivity of residual labelled DNA was measured. For this purpose, air-dried glass-fibre filters (MN85; Machery-Nagel, Düren, FRG) in a mixture containing Omnifluor (New England Nuclear) 4g/L of toluene were counted in a scintillation counter. The Cot 1/2 value was calculated by linear regression from 12 measurements and the correlation coefficient was >0.97 .

Results

Size of LDC genomes

Genome size for three LDC strains was determined by renaturation kinetics of fragmented DNA preparations of an average size of 10^3 base pairs. The figure shows the reassociation kinetics of the DNA obtained from LDC strains, compared with that of reference DNA from *E. coli*. The latter had a Cot 1/2 value of 1.123 and the corresponding values for LDC 15, 4 and A5 were 0.663, 0.563 and 0.312, respectively. The reassociation kinetics of three LDC strains are summarised in table II. They show that the LDC genomes were smaller than that of *E. coli* (4.2×10^6 bp). The values for DNA of LDC were in the range $(1.2\text{--}2.5) \times 10^6$ bp.

Figure. Kinetics of single-stranded DNA reassociation. Four samples of denatured bacterial DNA were re-annealed at 71°C, and reassociation kinetics was recorded as detailed in *Materials and methods*. Sources of DNA: *E. coli* (—x—), LDC 15 (—Δ—), LDC 4 (—○—) and LDC A5 (—□—).

Table II. Renaturation properties of DNA from LDC and *E. coli*

Test strain	Cot 1/2*	Genome size (bp $\times 10^6$)
<i>E. coli</i>	1.123	4.20
LDC 15	0.663	2.48
LDC 4	0.563	2.11
LDC A5	0.312	1.17

*The Cot 1/2 value was obtained by linear regression from 12 determinations with correlation coefficients higher than 0.97.

Homology amongst LDC genomes

Homologous and heterologous hybridisation of the genomes of LDC and *M. leprae* was made to explore their genetic relatedness. Table III shows that LDC strains 3, 4, 8 and A5 were very similar to each other with hybridisation values of 63.1–87.1%. The degree of homology of LDC 11 and 24, was lower than that of the preceding strains at 38–48%. LDC 15 was different. No LDC strain was similar to *M. leprae* with homologies of 0.3–18%.

Genetic relatedness of LDC and CMN organisms

To clarify the taxonomic position of LDC strains, their DNA was hybridised with those of reference CMN organisms. Table IIIB shows that there was homology of only 5–16% between three LDC strains and the two reference corynebacteria. LDC showed little homology with *M. bovis* and *N. caviae*. Among reference CMN bacteria, only *M. bovis* BCG was 46.6% homologous with *M. leprae*.

Discussion

The small genome of *M. leprae* (Clark-Curtiss *et al.*, 1985) may be due to the deletion of genes leading to nutritional deficiencies, which account for its inability to grow *in vitro*. Similar reasons may account for the inability of LDC, with genome sizes of $(1.2\text{--}2.5) \times 10^6$ bp, to grow in defined media

without serum supplements (Cocito and Deville, 1985).

LDC appear to be immunologically related to *M. leprae*. Indeed, sera from leprosy patients can recognise both organisms (Laub *et al.*, 1978). This relationship is accounted for by the cross-reactivity of *M. leprae* antigen 7 with the corresponding antigen M1 that is present in the cytoplasm and all wall of LDC (Gueur *et al.*, 1983). The unexpected finding that the genomes of LDC and *M. leprae* have similar base composition (G + C c. 56 mol %) (Danhaive *et al.*, 1982; Imaeda *et al.*, 1982; Hottat *et al.*, 1987) suggests that this may be reflected in a relationship at the genetic level. Although extensive homology between one LDC strain and *M. leprae* has previously been noted (Imaeda *et al.*, 1982), much lower hybridisation values were subsequently reported by the same authors (Athwal *et al.*, 1984). The results presented in table III show very limited hybridisation between the DNA of seven LDC strains and *M. leprae*. Since we have recently suggested that there is no homology between *M. leprae* and ADM, it can be inferred that *M. leprae*, LDC and ADM are genetically unrelated (De Kesel *et al.*, 1987; Hottat *et al.*, 1987 and unpublished results).

Previous work from our laboratory has shown that LDC strains, though having the characteristics of corynebacteria, because of the presence of cell-wall corynomycolic acid (Gailly *et al.*, 1982) and of DNA with G + C 56 mol % (Danhaive *et al.*, 1982),

Table III. Homology of the genomes of LDC, *M. leprae* and reference bacteria

Unlabelled DNA source	Relative hybridisations (mean $\pm$ SEM%)* with labelled DNA from			
	LDC 15	LDC A ₅	LDC 4	<i>M. leprae</i>
A				
<i>M. leprae</i>	0	0	0	100
LDC 3	4.1 $\pm$ 2.6	70.8 $\pm$ 14.4	87.1 $\pm$ 1.3	2.8 $\pm$ 4.0
LDC 4	6.5 $\pm$ 1.8	75.4 $\pm$ 9.6	100	6.4 $\pm$ 9.1
LDC 8	9.6 $\pm$ 4.7	63.1 $\pm$ 7.6	71.5 $\pm$ 1.1	8.8 $\pm$ 6.0
LDC 11	9.7 $\pm$ 5.3	48.0 $\pm$ 5.7	46.6 $\pm$ 5.3	14.0 $\pm$ 11.5
LDC 15	100	12.3 $\pm$ 3.7	13.6 $\pm$ 2.8	8.3 $\pm$ 7.1
LDC 24	3.6 $\pm$ 5.1	37.8 $\pm$ 16.0	43.5 $\pm$ 19	17.9 $\pm$ 24.2
LDC A ₅	4.7 $\pm$ 5.7	100	75.0 $\pm$ 1.1	0.3 $\pm$ 0.4
B				
<i>C. diphtheriae</i>	7.7 $\pm$ 1.4	12.2 $\pm$ 1.9	5.2 $\pm$ 6.7	21.7 $\pm$ 9.2
<i>C. xerosis</i>	5.2 $\pm$ 0.04	16.5 $\pm$ 3.2	11.0 $\pm$ 3.2	9.2 $\pm$ 1.4
<i>M. bovis</i> BCG	8.4 $\pm$ 3.1	12.3 $\pm$ 5.0	5.1 $\pm$ 4.7	46.6 $\pm$ 17.0
<i>N. caviae</i>	3.0 $\pm$ 4.2	8.2 $\pm$ 7.5	1.8 $\pm$ 0.7	7.7 $\pm$ 0.8
<i>E. coli</i>	0	0	0	0

* Each experiment was performed at least three times.

cross-reacted immunologically with mycobacteria rather than with *Corynebacterium* spp. (Ridell, 1977; Laub *et al.*, 1978). These observations and the lack of genetic relatedness between LDC and reference corynebacteria (table III) have rendered the taxonomic position of LDC uncertain. The taxonomic position of *M. leprae* is also rendered uncertain by a base content of G+C 56 mol % (Imaeda *et al.*, 1982; Hottat *et al.*, 1987) rather than 62–70 mol % of typical mycobacterial DNA, and replacement of L-alanine by glycine in the tetrapeptide of peptidoglycan (Draper, 1976).

We have reported previously the homogeneity of the LDC group. Isolates from patients worldwide had similar cell-wall composition, immunological properties and DNA base composition (Danhaive *et al.*, 1982; Cocito and Delville, 1983). This

homogeneity is suggested by the presence in all LDC strains of a specific GA^mTC genetic marker (A^m = N⁶ methyladenine), in which the adenine of GATC sequences of DNA is replaced by 6-methyladenine, a feature not shared by any organisms of the CMN group tested (Hottat *et al.*, 1987). However, the results reported have indicated some heterogeneity within the LDC group. Different isolates were found to differ in genome size (table II) and homology levels (table III).

We conclude that the genomes of LDC strains are small and that they are genetically unrelated to conventional corynebacteria and *M. leprae*. On the basis of the present and previous results, we propose that LDC, because of their unusual properties, represent a new species within the CMN group of micro-organisms.

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