

Restriction Fragment Length Polymorphisms of the DNA of Selected *Naegleria* and *Acanthamoeba* Amebae

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Fourteen strains of *Naegleria fowleri*, two strains of *N. gruberi*, and one strain each of *N. australiensis*, *N. jadini*, *N. lovaniensis*, *Acanthamoeba* sp., *A. castellanii*, *A. polyphaga*, and *A. comandoni* isolated from patients, soil, or water were characterized by restriction fragment length polymorphisms. Total cellular DNA (1 µg) was digested with either *Hind*III, *Bgl*III, or *Eco*RI; separated on agarose gels; and stained with ethidium bromide. From 2 to 15 unusually prominent repetitive restriction fragment bands, totaling 15 to 50 kilobases in length and constituting probably more than 30% of the total DNA, were detected for all ameba strains. Each species displayed a characteristic pattern of repetitive restriction fragments. Digests of the four *Acanthamoeba* spp. displayed fewer, less intensely staining repetitive fragments than those of the *Naegleria* spp. All *N. fowleri* strains, whether isolated from the cerebrospinal fluid of patients from different parts of the world or from hot springs, had repetitive restriction fragment bands of similar total lengths (ca. 45 kilobases), and most repetitive bands displayed identical mobilities. However, polymorphic bands were useful in identifying particular isolates. Restriction fragment length polymorphism analysis generally was consistent with taxonomy based on studies of infectivity, morphology, isoenzyme patterns, and antibody reactivity and suggests that this technique may help classify amebae isolated from clinical specimens or from the environment.

Restriction fragment length polymorphisms have been useful in the classification of viruses (4), bacteria (6), bacterial plasmids (9), and eucaryotic pathogens (13). Restriction fragment length polymorphism analysis also has been used to determine the origins of particular pathogenic isolates (5). For most bacteria and eucaryotes, restriction enzyme digestion of the total cellular DNA produces smears or banding patterns that are too complex for easy analysis (7). To simplify the banding patterns analyzed, separated DNA usually is transferred to membranes and hybridized with cloned DNA that detects particular genomic sequences, especially repetitive sequences. For eucaryotes, repetitive DNA represents a good marker for strain characterization, because the primary sequence, chromosomal location, and copy number change rapidly for many classes of repetitive DNA through evolutionary time; these variations are reflected by changes in restriction fragment length polymorphism patterns among particular strains (12).

As part of our research on the characterization of pathogenic and nonpathogenic free-living amebae of the genera *Acanthamoeba* and *Naegleria*, which contain strains that are opportunistic human pathogens, we isolated the total genomic DNA of several strains and digested the DNA with restriction endonucleases. After the DNA was electrophoresed on agarose gels and stained with ethidium bromide, we noticed strikingly abundant, well-defined DNA fragments when the gels were examined under UV illumination. The patterns of these DNA fragments may be useful in characterizing the pathogenic and nonpathogenic *Naegleria* and *Acanthamoeba* species.

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MATERIALS AND METHODS

Amebae. Strains of *Naegleria* and *Acanthamoeba* analyzed are listed in Table 1 along with their places of origin and years of isolation. All strains were grown for 4 to 5 days and harvested at late log phase. *N. fowleri*, *N. gruberi*, and *N. jadini* were grown in modified Nelson medium; *N. australiensis* was grown in TYPH medium; and *Acanthamoeba* was grown in PYG medium, as described previously (14). *N. gruberi*, *N. jadini*, *A. castellanii*, *A. polyphaga*, and *A. comandoni* were incubated at 24°C; all other amebae were grown at 37°C.

DNA preparation. Pellets of amebae (7×10^6 to 2×10^7 cells) were lysed and digested by incubation in 5 ml of 1% sodium dodecyl sulfate–5 mM EDTA–0.3 mg of proteinase K per ml–50 mM Tris (pH 8.0) for 2 h at 37°C. Equivolume phenol saturated with 1 mM EDTA–10 mM Tris (pH 8.0) was added, and samples were thoroughly mixed by gentle inversion for 5 min at room temperature. Samples were chilled for 5 min on ice and were then centrifuged at $4,000 \times g$ for 10 min at 4°C. The aqueous phase was removed, and 0.1 volume of 3 M sodium acetate (pH 5.4) and equivolume –20°C isopropanol were added with mixing (1). After storage at –20°C for 20 min to overnight, samples were centrifuged at 4°C at $10,000 \times g$ for 20 min. The pellet was washed with 1 ml of 70% ethanol containing 0.1 mM EDTA–1 mM Tris (pH 7.6) and once with 95% ethanol, dried for 10 min at 50°C, and dissolved in 0.5 ml of 1 mM EDTA–10 mM Tris (pH 7.8).

Enzyme digestion and electrophoresis. DNA samples (1 to 7 µg, 15 µl) were digested for 1 h at 37°C with *Bgl*III, *Hind*III, or *Eco*RI in 50 µl of corresponding restriction enzyme buffers (International Biotechnologies, Inc.). Samples (20 µl) were electrophoresed at 4 V/cm in 0.7% flat-bed agarose gels without ethidium bromide. Gels were stained for 30 min with 1 µg of ethidium bromide per ml, destained for 10 min in H₂O, and photographed under shortwave UV illumination (8).

Southern blot analysis. Gels were incubated for 20 min in

TABLE 1. Origins and sources of selected strains of amoebae used in this study

Ameba	Strain	Place and yr of origin	Host or source
<i>N. fowleri</i>	CA-66	Australia (1966)	Human
	HB-3	Czechoslovakia (1968)	Human (CSF) ^a
	HB-1	Florida (1968)	Human (CSF)
	NH-1	New Zealand (1972)	Human (CSF)
	HB-5	Texas (1977)	Human (CSF)
	HBWS-1	Georgia (1977)	Human (CSF)
	MP	California (1978)	Human (CSF)
	A 5086	Texas (1979)	Human (CSF)
	MD	California (1980)	Human (CSF)
	NY	New York (1980)	Human (CSF)
	CDC:0784:1	Texas (1984)	Human (CSF)
	CDC:0784:2	Texas (1984)	Human (CSF)
	CDC:0487:1	Arizona (1987)	Human (CSF)
	CDC:0687:1	New Mexico (1987)	Hot springs
	<i>N. australiensis</i>	ATCC 30958	Australia (1973)
<i>N. lovaniensis</i>	76-15-250	Belgium (1976)	Thermally polluted water
<i>N. jadini</i>	0400	Belgium (1971)	Private swimming pool
<i>N. gruberi</i>	NB-1 (=1518/1A)	United Kingdom (pre-1950)	Soil
	Eg	California (1959)	Soil
<i>Willaertia</i> sp.	CDC:0687:6	New Mexico (1987)	Hot springs
<i>A. castellanii</i>	ATCC 30011	United Kingdom (1930)	Yeast culture
<i>A. polyphaga</i>	ATCC 30871	Wisconsin (1964)	Soil
<i>A. comandoni</i>	ATCC 30135	France (1935)	Soil
<i>Acanthamoeba</i> sp.	CDC:0187:1	Massachusetts (1987)	Human (cornea)

^a CSF, Cerebrospinal fluid.

0.5 M NaOH containing 1.5 M NaCl, neutralized in 3 M sodium acetate (pH 5.4), and transferred to nylon filters as previously described (10). After the filters were baked at 80°C for 1 h, they were blocked and hybridized with a nick-translated plasmid (7E3; courtesy of Thomas Byers, Ohio State University) containing 5.2 kilobases of *A. castellanii* mitochondrial DNA. Filters were also hybridized to Agr1 2A, a ribosomal DNA plasmid isolated from *Anopheles* sp. (2). Specific activities were 10⁸ cpm/μg of DNA. Filters were washed at 55°C in 2× SSC containing 0.1% sodium dodecyl sulfate (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y.) was exposed with two enhancing screens for 10 to 60 h at -80°C.

RESULTS

Ethidium bromide-stained gels. Figures 1 through 3 show typical patterns of ethidium bromide-stained gels. For *N. fowleri* and *N. gruberi*, prominent simple bands totaling about 45 kilobases in molecular weight were clearly detectable above the genomic background smear. If a sufficient amount of restriction enzyme was used to achieve full digestion (about 7 U of enzyme per μg of DNA), the background genomic smear would be almost undetectable and no high-molecular-weight DNA would remain near the origin. In some partial digestions, evenly spaced bands were detectable between the position of the genomic smear and the highest-molecular-weight band (Fig. 3). A total of 7 to 10 remarkably prominent bands were resolvable for *N. fowleri* and *N. gruberi* after *EcoRI* digestion, whereas *N. australiensis* showed only 2 to 3 prominent bands. Each species showed a recognizable pattern of prominent bands with the three restriction enzymes used. Also, particular geographic isolates of *N. fowleri* differed slightly in their banding patterns after *EcoRI* digestion.

Band characterization. The stained bands were sensitive to DNase but not to RNase; without restriction enzyme diges-

tion, only one small band below the genomic smear was detected (data not shown). The *A. castellanii* mitochondrial plasmid 7E3 hybridized to this dim band for *A. castellanii* and to a single 7.8-kilobase band of *EcoRI*-digested *A.*

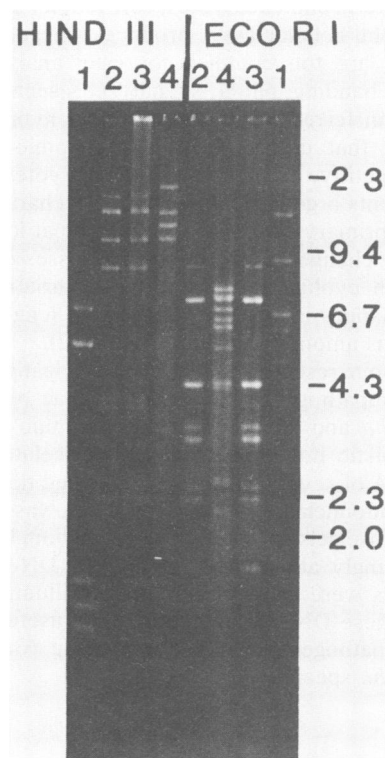


FIG. 1. Agarose gel profiles of *HindIII* and *EcoRI* digestions of genomic DNA of *N. gruberi* Eg (lane 1), two strains of *N. fowleri* (HBWS [lane 2] and A 5086 [lane 3]), and *N. lovaniensis* 76-15-250 (lane 4).

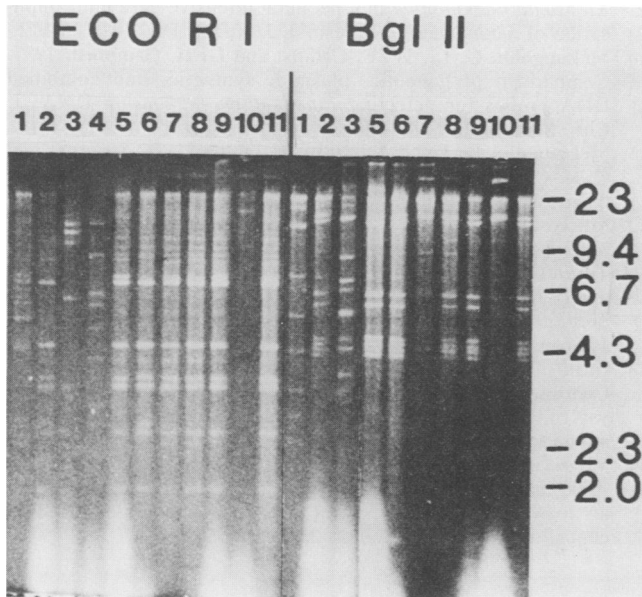


FIG. 2. Agarose gel profiles of *EcoRI* and *BglII* digestions of genomic DNA of *N. lovaniensis* 76-15-250 (lane 1); seven strains of *N. fowleri* (A 5086 [lanes 2 and 6], CDC:0784:1 [lane 5], NY [lane 7], HB-5 [lane 8], MD [lane 9], CDC:0784:2 [lane 10], HB-3 [lane 11]); and two strains (NB-1 [lane 3] and Eg [lane 4]) of *N. gruberi*. The profile of *BglII* digestion of *N. gruberi* Eg DNA is not shown.

castellani DNA; the detected band corresponded in mobility to one of the prominent bands of *EcoRI*-digested *Acanthamoeba* DNA. The *Acanthamoeba* mitochondrial probe did not cross-hybridize to any DNA of the other amoeba strains analyzed; similarly, the heterologous ribosomal probe did not hybridize to any of the prominent bands of *Naegleria* or *Acanthamoeba* species, even with low-stringency washes (45°C, 2× SSC).

DISCUSSION

We are unaware of any other eucaryotic organism that displays so prominent a class of DNA bands after restriction enzyme digestion. The underlying genetic reason for the remarkably simple banding pattern obtained with restriction enzyme digestion of total amoeba DNA remains unclear. With complete digestion, the complex genomic smear was virtually invisible, suggesting that the DNA in the prominent bands probably contains more than 30% of the total DNA of the organism. The homologous *Acanthamoeba* mitochondrial probe, but not the heterologous ribosomal probe, hybridized to one of the prominent bands, suggesting that these prominent bands may represent amplified mitochondrial rather than ribosomal genes. The nature of these remarkably abundant bands awaits clarification through additional cloning, hybridization with more closely related probes, and sequencing approaches.

The relative similarity of DNA banding patterns between *N. fowleri* strains and the differences in the patterns between *N. fowleri* and other *Naegleria* spp. observed in this study correlated well with the results obtained previously by using such criteria as pathogenicity, isoenzyme patterns, and monoclonal antibody typing (14).

De Jonckheere recently described prominent ethidium bromide-stained DNA bands after restriction enzyme digestion of *Willaertia magna*, *Didascalus thorontonii*, and *Naegleria* spp. (3).

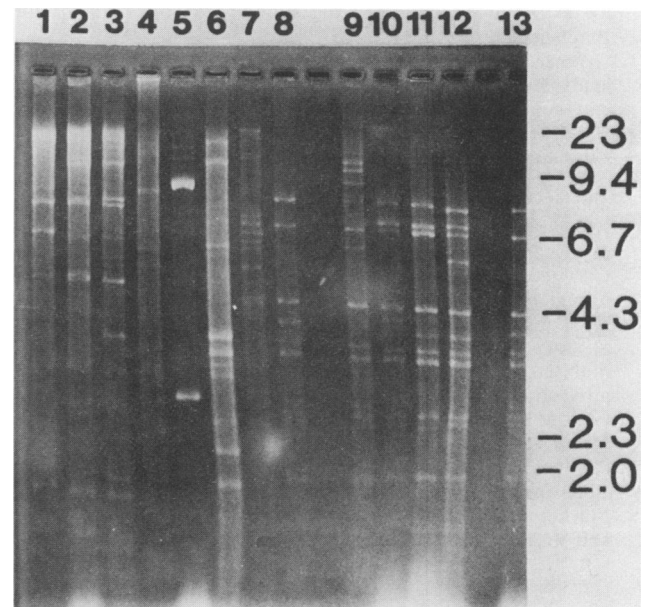


FIG. 3. Agarose gel profiles of *EcoRI* digestion of genomic DNA of one strain each of *A. polyphaga* ATCC 30,871 (lane 1), *A. castellanii* ATCC 30,011 (lane 2), *Acanthamoeba* sp. strain CDC: 0187:1 (lane 3), *A. comandoni* ATCC 30,135 (lane 4), *N. australiensis* (lane 5), *N. jadini* 0400 (lane 6), *N. lovaniensis* (lane 7), and *N. gruberi* Eg (lane 9) and of five strains of *N. fowleri* (CDC:0687:1 [lane 8], CA-66 [lane 10], MP [lane 11], NH-1 [lane 12], and CDC: 0487:1 [lane 13]).

Although the procedures for DNA isolation differed in the two studies, the major *EcoRI* and *HindIII* bands of particular *Naegleria* strains correspond in mobility. However, the bands on our gels were typically more prominent, with less genomic DNA remaining near the origin and a dimmer genomic smear in each lane. In our hands, resolution of more intense and distinct bands depended on gentle mixing during DNA isolation, enough restriction enzyme, low voltage (below 10 V/cm) during electrophoresis, and omission of ethidium bromide during the gel run.

In summary, analysis of restriction fragment length polymorphisms of totally digested genomic DNA allows one to reliably identify particular amoeba species and strains. This finding suggests that oligonucleotide analysis may be useful in studies on the molecular epidemiology and ecology of small free-living and pathogenic amoebae. If a subset of the sequences of these simple, abundant, repetitive sequences is characteristic of a given pathogenic strain, the development of rapid dot blot hybridization assays may be feasible (11).

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