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Acanthamoeba healyi N. Sp. and the Isoenzyme and Immunoblot Profiles of *Acanthamoeba* spp., Groups 1 and 3

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ABSTRACT. Two strains of *Acanthamoeba* isolated from human brain tissue and a strain of *Acanthamoeba* isolated from a fish were compared with 10 species of *Acanthamoeba* belonging to groups 1, 2 and 3 based on their isoenzyme profiles and antigenic characteristics. A total of 12 enzymes were studied. The isoenzymes and antigens were electrophoretically separated on polyacrylamide gradient gels, and the patterns obtained were compared after appropriate staining for particular enzymes and reactivities with homologous and heterologous rabbit anti-*Acanthamoeba* antisera. One of the human strains (CDC:1283:V013) was identified as *A. healyi* n. sp. because of its unique isoenzyme profiles for 11 of the 12 enzymes tested. The other human isolate was reidentified as *A. culbertsoni* because its isoenzyme profiles for 10 of 12 enzymes resembled those of *A. culbertsoni*, Lilly A-1 strain. Since the isoenzyme profiles and the antigenic patterns of the fish isolate as well were remarkably similar to those of *A. royreba*, it was considered as a strain of *A. royreba*. Polyacrylamide gradient gel electrophoresis appears to be a powerful technique for the study of isoenzymes and antigens of *Acanthamoeba*.

Key words. AIDS, SDS-PAGGE, taxonomy, zymodemes.

MEMBERS of the genus *Acanthamoeba* are ubiquitous fresh water and soil amebae that occur world-wide. During the last decade, these amebae have been isolated with increasing frequency from contact lens paraphernalia as well as from human tissue such as corneal scrapings and the central nervous system [9, 22]. *Acanthamoeba* can be readily recognized because of the striking morphologic characteristics of the trophozoites and cysts. Based on the size and morphologic features of cysts, Pussard & Pons [15] established 18 species and placed them in three groups. Group 1 now consists of four species (*A. astronyxis, A. comandoni, A. echinulata,* and *A. tubiashi*) characterized by large trophozoites and cysts (≥ 18 μm) [14, 19]. Species in groups 2 and 3 are smaller (≤ 18 μm) but can be separated from each other on the basis of cyst morphology. Group 2 comprises 10 species (*A. castellanii, A. polyphaga, A. rhysodes, A. mauritaniensis, A. divionensis, A. griffini, A. lugdunensis, A. quina, A. hatchetti,* and *A. triangularis*), and five species (*A. culbertsoni, A. lenticulata, A. palestinensis, A. pustulosa,* and *A. royreba*) are in group 3 [14, 19].

Although cyst characteristics make genus identification easy, differentiation to the species level is difficult. This is especially true for members of groups 2 and 3 which cannot always be identified by species on a morphologic basis alone. As a result, the most recent identification attempts have been based on both morphologic and biochemical criteria such as isoenzymes, antigens, protein profiles, and restriction fragment-length polymorphism analysis of DNA resolved by electrophoretic methods [2, 6, 14, 19]. Cladistic analysis of the data has also been used to elucidate the affinities of the various species of *Acanthamoeba* [4].

This paper presents the methods used to isolate *Acanthamoeba* from the CNS of humans and identify them to the species level based on morphologic characteristics and isoenzyme and antigenic profiles. We also describe the usefulness of polyacrylamide gradient gel electrophoresis (PAGGE) in the resolution of isoenzymes and antigens of groups 1 and 3 *Acanthamoeba* spp. and its applicability to the taxonomy of this protozoan.

MATERIALS AND METHODS

Case 1. A 7-year-old girl from Barbados with headache, weakness of the right upper and lower extremities, several episodes of vomiting, and positive Babinski sign on the right was admitted to the hospital. Computed tomography (CT) showed a large mass in the left parietal area. A craniotomy was performed, and histopathology of the excised tumor-like mass revealed amebic trophozoites [13]. A small piece of the biopsied brain tissue was frozen and sent to the Centers for Disease Control, where *Acanthamoeba* (CDC:1283:V013) was isolated on non-nutrient agar plates seeded with *Escherichia coli* and MRC human lung cell culture [13]. Based on cyst morphology and reactivities of the amebae in the brain sections with the rabbit anti-*A. palestinensis* serum in the indirect immunofluorescence (IIF) test, the amebae were identified at that time as *A. palestinensis* [13].

Case 2. A 34-year-old man with AIDS was admitted to the hospital with numerous painful, widely distributed skin lesions, hemiparesis of the right side, and clonic movement of the right arm [23]. A CT scan showed three walnut-sized, lucent lesions in the left cerebral cortex. Histopathology of the biopsied left frontal brain revealed acute necrotizing encephalitis but no microorganisms. The patient died the next day. At autopsy, amebic trophozoites and cysts were seen in the brain as well as in the skin lesions [23]. Amebic organisms from brain tissue were isolated in human embryonic kidney (HEK) cell culture and identified as *A. culbertsoni* (CDC:0884:V021) on the basis of morphology and reactivities of the amebae in the tissue sections with rabbit antiserum to *A. culbertsoni* in the IIF assay [23].

***Acanthamoeba* strains.** The 13 strains of *Acanthamoeba* used in this study are listed in Table 1, along with the history of their isolation and growth temperature. They include the five species belonging to group 3; four species belonging to group 1; *A. castellanii,* a representative of group 2; two strains of amebae (CDC:1283:V013 and CDC:0884:V021) isolated from the brain tissue of patients with *Acanthamoeba* encephalitis; and a strain of ameba (CDC-Fish-SK) with a history of isolation from a fish in South Korea. This isolate was submitted to one of us (GSV) by Dr. James Yang on agar plate with *E. coli* and subsequently axenized at the Centers for Disease Control. All strains of amebae were grown initially on non-nutrient agar plates with *E. coli* and then axenically grown in a proteose peptone-yeast extract-glucose medium as described previously [20], except for the addition of 5% fetal bovine serum, in 150-cm² Falcon plastic tissue culture flasks.

Preparation of cell pellets. Trophozoites in log-phase of growth were harvested after 2–4 days of axenic culture at the optimum temperature (Table 1). The supernatants were discarded, and 50 ml of WB saline [20] was added to the flasks containing attached trophozoites. After the flasks were chilled on ice for 5–10 min, the solution was transferred to 50-ml Falcon plastic

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Table 1. Strains of *Acanthamoeba* spp. used in this study.

Lane No.	Species/strain Designation	Isolated from	Temp. (°C)	Ref.
1	CDC:0884:VO21	Human brain, USA	37	23
2	CDC:1283:VO13	Human brain, USA	37	13
3	*A. culbertsoni* ATCC 30171	Tissue culture, USA	37	14
4	*A. palestinensis* ATCC 30870	Soil, Israel	30	14
5	*A. pustulosa*[a] Ge3a	Water, France	30	15
6	*A. lenticulata* ATCC 30841	Water, France	37	14
7	*A. royreba* ATCC 30884	Tissue culture, USA	37	14
7A	CDC-Fish-SK[b]	Fish tissue, South Korea	37	
8	*A. castellanii* ATCC 30011	Yeast culture, England	30	14
9	*A. astronyxis* ATCC 30137	Soil, USA	30	14
10	*A. tubiashi* ATCC 30867	Water, USA	30	8
11	*A. comandoni* ATCC 30135	Soil, France	30	14
12	*A. echinulata*[a] 378	Water, France	30	15

[a] Obtained from Dr. Marc Pussard.
[b] Obtained from Dr. James Yang.

tubes, which were then centrifuged at 563 *g* for 10 min at 4° C. The cells were washed twice as above and counted using a hemocytometer. To every 2×10^8 cells, 0.8 ml of enzyme-stabilizing solution containing 1 mM each of dithiothreitol, 6-amino caproic acid, and disodium EDTA [16] was added, and the pellets were stored in liquid nitrogen until used.

Extract preparation and SDS-treatment. *Acanthamoeba* cell pellets were retrieved from liquid nitrogen and processed. For enzymatic studies, amebae were lysed by three cycles of freezing and thawing, 5 min per cycle, in a dry ice-methanol slurry and a 37° C water bath. The suspension was centrifuged at 24,000 *g* for 30 min at 4° C, and the water-soluble supernatant containing native proteins was aliquoted and stored in liquid nitrogen. For antigenic studies, the pellets were sonicated using a W-375 Sonifier (Heat Systems-Ultrasonics Inc., Plainview, NY) at 70% power, 10% duty cycle, pulse mode, for 3 min at 4° C in a solution (TBE) containing 81.2 mM Tris(hydroxymethyl) aminomethane (Tris), 23 mM boric acid, and 1.5 mM EDTA. The sonicated extracts were centrifuged at 24,000 *g* for 30 min to remove particulate matter and cell debris. The supernatants were collected, aliquoted, and stored in liquid nitrogen. A protein determination was done for each extract using the method of Bradford [1]. The dye reagent was purchased from Bio-Rad Laboratories (Richmond, CA), and a suspension of albumin and globulin from Sigma (St. Louis, MO) was used as protein standard.

For isoenzyme analysis, equal volumes of a solution containing 80 μg bromophenol blue in 30% sucrose were added to each extract of native proteins just before electrophoresis. For silver staining and immunoblots, the extracts were treated with a solution containing 10% sodium dodecyl sulphate (SDS), 9 M urea, and 0.05 M Tris hydrochloride, pH 8, to obtain a final concentration of 2.5% SDS, 2.25 M urea, and 1 μg of protein/μl. Tracking dye solution (50 mg bromophenol blue, 8 ml glycerol, 1 ml 0.05 M Tris hydrochloride, pH 8.0, and 1 ml deionized water) was used in a 3% concentration after the samples were heated for 15 min at 65° C in a water bath.

High-resolution PAGGE and buffer systems. All PAGGE reagents were purchased from Bio-Rad, except where noted. Conditions for PAGGE were as previously reported [11, 18], with some modifications. Briefly, for isoenzyme electerophoresis we used 3–20% gradient gels with a 3% stacking gel; the gels measured 200 × 180 × 1.5 mm. For silver staining and immunoblot procedures, we used 5–20% gradient gels with a 3% stacking gel; the gels measured 80 × 180 × 0.75 mm.

A continuous and a discontinuous buffer system were used in the study. The continuous system, TBE, was used for enzymes. The discontinuous system, used for SDS-treated proteins, consisted of TBE in the lower chamber and a freshly prepared solution of 41 mM Tris, 40 mM boric acid, and 0.1% (w/v) SDS in the upper chamber.

A Pharmacia vertical electrophoresis apparatus GE-2/4 LS (Pharmacia LKB Biotechnology, Piscataway, NJ) was used for isoenzyme electrophoresis, which was performed at least four times for each strain using extracts prepared from different harvests to check for fidelity and reproducibility of the patterns. Optimum concentration of proteins necessary for the visualization of particular enzymes was also determined. For enzyme separation, the gels were pre-electrophoresed for 45 min at 250 V constant. Constant amounts of protein for each enzyme (Table 2) were loaded and electrophoresed at 250 V maximum. After 60 min the voltage was increased to 500 V maximum. The power was turned off after 3 h, when the blue tracking dye could no longer be seen at the bottom of the gel.

In the discontinuous system, the SDS-treated proteins were loaded at a concentration of 0.25 μg/mm of lane width for silver stain and 0.75 μg/mm lane width for immunoblot. Unstained molecular weight standards, a 1:1 mixture of high molecular weight standards (Bio-Rad) and low molecular weight standards (Pharmacia) were used to calibrate relative molecular weights of the resolved bands. For immunoblots, 0.5 μl/mm of prestained protein standards (Bethesda Research Laboratories, Gaithersburg, MD) was also used. A constant current of 7.5 mA per gel was applied during the first 20 min and then increased to 15 mA per gel. Voltage was allowed to float, and the wattage was limited to 25 W per gel. The power was turned off after 1 h and 30 min of electrophoresis, 15 min after the tracking dye migrated away from the gel. A constant temperature of 4° C for enzymes and 7° C for SDS-treated proteins was maintained during electrophoresis.

Developing solutions for enzymes. All reagents used for isoenzyme study were purchased from Sigma, except where noted. The enzymes used in this study are listed in Table 2 along with the amount of protein loaded and details for developing solutions [5, 10, 11]. All enzymes except ES were developed in the dark at 37° C. After enzyme development, the gels were immersed in a solution of 20% methanol and 5% acetic acid and photographed immediately using a Polaroid camera, model 545. Substrate control gels for each enzyme tested were immersed in the same developing solution, but substrate was omitted.

Silver stain and immunoblot assays. Separated SDS-treated proteins were either silver stained [18] or electrophoretically transferred to Immobilon membranes with a 0.2-μm pore size (Millipore Corporation, Bedford, MA). Electrophoresis was performed using a Trans-blot cell and a power supply model 200/2.0 (Bio-Rad) at a constant voltage of 90 V for 1 h and 30 min. The current increased from 1.05 A at the beginning to 2.0 A at the end of electrophoresis and the buffer temperature increased from 10° C at the beginning to 35° C at the end [17]. After

Table 2. Staining reagents/100 ml buffer used for visualizing *Acanthamoeba* enzymes.

Enzyme[a]	Buffer	Substrate	Coenzyme[b] (mg)	Dye[c] (mg)	Other Reagents	μg/lane[d]
Alcohol dehydrogenase ADH (EC 1.1.1.1)	0.075 M Tris/HCl, pH 7.1	N-Octanol, 1 ml	NAD 20	MTT 15 PMS 2	0.005 M NaCN	80
Malate dehydrogenase MDH (EC 1.1.1.37)	0.1 M Tris/HCl, pH 8.5	1 M L-Malic acid, 1 ml adjusted to pH 7.3	NAD 15	MTT 12 PMS 4	—	10
Malic enzyme ME (EC 1.1.1.40)	0.1 M Tris/HCl, pH 7.4	1 M L-Malic acid, 0.3 ml adjusted to pH 7.3	NADP 20	MTT 20 PMS 4	0.01 M $MgCl_2$	20
Isocitrate dehydrogenase IDH (EC 1.1.1.42)	0.05 M Tris/HCl, pH 8.5	Isocitric acid, 20 mg	NADP 20	MTT 20 PMS 4	0.005 M $MgCl_2$	30
Glucose-6-phosphate dehydrogenase Gd (EC 1.1.1.49)	0.1 M Tris/HCl, pH 8.0	Glucose-6-phosphate, 20 mg	NADP 10	MTT 15 PMS 10	0.02 M $MgCl_2$	40
Hexokinase HK (EC 2.7.1.1)	0.1 M Tris/HCl, pH 7.4	Glucose, 190 mg	ATP 24 NADP 24	MTT 24 PMS 4	0.01 M $MgCl_2$	40
Phosphoglucomutase PGM (EC 2.7.5.1)	0.1 M Tris/HCl, pH 7.4	Glucose-1-phosphate, 100 mg	NADP 10	MTT 20 PMS 4	0.01 M $MgCl_2$	25
Esterase ES (EC 3.1.1.1)	0.1 M phosphate, pH 6.5	α-naphthyl acetate, 150 mg in 40:60 acetone : water (v/v)	—	Fast blue RR 150	—	25
Alkaline phosphatase ALP (EC 3.1.3.1)	0.15 M Tris/citrate, pH 8.45	α-naphthyl phosphate, 100 mg	—	Fast blue BB 100	0.001 M $MgCl_2$ 0.0025 M $MnCl_2$ polyvinyl pyrrolidone 200 mg	15
Acid phosphatase ACP (EC 3.1.3.2)	0.05 M citrate, pH 4.5	α-naphthyl phosphate, 100 mg	—	Fast blue BB 100	—	15
Leucine aminopeptidase LAP (EC 3.4.1.1)	0.2 m Tris/maleate, pH 6.0	L-leucine-β-naphthyl amide, 40 mg	—	Fast black K salt 50	—	80
Glucose phosphate isomerase GPI (EC 5.3.1.9)	0.1 m Tris/HCl, pH 8.0	D-Fructose-6-phosphate, 36 mg	NADP 10	MTT 10 PMS 4	0.01 M $MgCl_2$ Glucose-6-phosphate dehydrogenase, 20 units	8

[a] EC, Enzyme Commission number.
[b] NAD, Nicotinamide adenine denucleotide; NADP, Nicotinamide adenine dinucleotide phosphate.
[c] MTT, 3-(4,5-dimethyl thiazolyl)-2,5-diphenyl tetrazolium bromide; PMS, phenazine methosulfate.
[d] μg of protein applied in each lane of the gel.

transference, the membranes were washed four times for 5 min each in phosphate-buffered saline-Tween (PBS-T, 0.01 M PBS, pH 7.2, containing 0.3% Tween 20). After the first wash in PBS-T, strips containing the unstained molecular weight standards were cut and stained with Aurodie (Janssen, Newton, MA). Prebleed control rabbit sera and rabbit sera containing antibodies against whole trophozoites of *A. culbertsoni, A. palestinensis, A. royreba, A. astronyxis, A. comandoni, A. castellanii* or CDC:1283:V013, were diluted 1:1,000 in PBS-T and used to probe the resolved antigens. The reaction was allowed to occur overnight at 4° C on a rocker platform [18]. The membranes were then washed four times, 5 min per wash, in PBS-T. Peroxidase-labeled goat anti-rabbit IgG (Cappel, Westchester, PA) at a dilution of 1:1,000 in PBS-T was added to the sheets and incubated for 1 h at room temperature. After three washes in PBS-T and a final wash with PBS only, the membranes were developed for 15 min in a solution containing 5 mg of 3,3'-diaminobenzidine tetrahydrocloride (DAB) and 10 μl of 30% H_2O_2 in 100 ml of PBS, pH 7.2. They were then washed with distilled water and dried at room temperature.

Preparation of antisera. Antibodies to the various Acanthamoeba species (*A. astronyxis, A. comandoni, A. castellanii, A. culbertsoni, A. palestinensis, A. royreba, A. lenticulata,* and CDC:1283:V013) were produced in rabbits by multiple intravenous injections of washed trophozoites and cysts from culture as described previously [21].

RESULTS

Isolation of ameba from brain tissue. Agar plates inoculated with thawed and minced brain tissue from Case 1 revealed growth of amebae within 72 h and the amebae began to differentiate into cysts after 7 days. However, in the MRC lung cell culture, amebic growth became noticeable only after 9 days of brain tissue incubation, when foci of cleared plaques began to appear in the monolayer. The cell culture was totally destroyed within 15 days, resulting in a monolayer of amebae and cysts. Morphologic analysis of the trophozoites and cysts indicated that the amebae belonged to group 3. The trophozoites (Fig. 1A) measured 20.4–42 μm long (mean, 32.6 μm) and 16.7–24 μm wide (mean, 18.9 μm) when grown on an agar plate. Feeding form was variable in shape and size, with a wide, clear, hyaline anterior zone of protoplasm. Cysts (Fig. 1B) were oval to round, with a relatively thick, gently rippled ectocyst and a well-developed round or irregular endocyst. The cysts ranged from 10.5 to 18.0 μm (mean, 14.1 μm) in diameter.

Isoenzymes. Isoenzyme profiles for 8 of the 12 enzymes studied are shown in Fig. 2 and 3, and the corresponding lane number for each species is given in Table 1. All substrate control gels

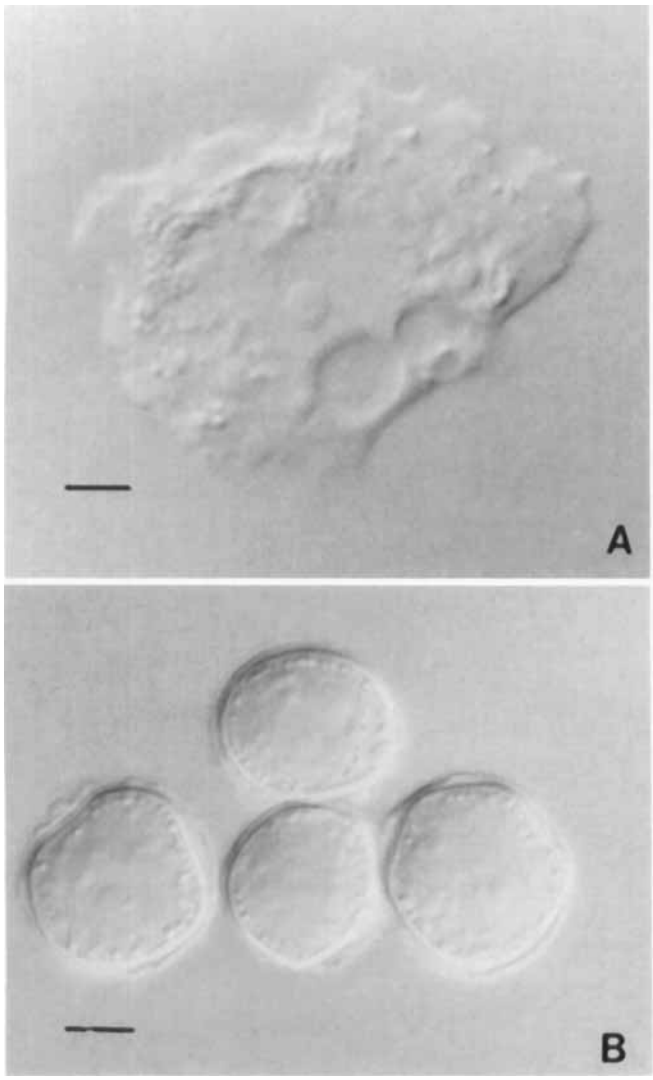

Fig. 1. Photomicrographs of **A**) trophozoite and **B**) cyst of *A. healyi*; differential interference contrast. Bar = 10 μm.

(not shown) showed no visible enzymatic activity except where noted for MDH (Fig. 3D). All isozyme patterns were consistent in all runs.

Among the four species of group 1, *A. astronyxis, A. comandoni,* and *A. tubiashi* exhibited distinctly different patterns for all 12 enzymes. *Acanthamoeba echinulata* also had unique profiles for eight isoenzymes: ES, PGM, ACP, Gd, ME, IDH (Fig. 2, 3), GPI and ALP (not shown) but resembled *A. comandoni* in the HK, MDH (Fig. 2, 3), LAP, and ADH (not shown) patterns.

All five species included in group 3 showed unique ES, PGM, ACP, Gd, LAP, ALP, and ME patterns (Fig. 2, 3). *Acanthamoeba culbertsoni, A. palestinensis,* and *A. pustulosa* had unique HK profiles, but the HK profiles for *A. lenticulata* and *A. royreba* were similar (Fig. 2). *Acanthamoeba culbertsoni, A. lenticulata,* and *A. royreba* had unique IDH, MDH, ADH, and GPI isozyme profiles that differed not only from one another but also from those of *A. palestinensis* and *A. pustulosa,* whose IDH, MDH (Fig. 3), ADH, and GPI (not shown) patterns were similar.

CDC:1283:V013, which was originally identified as *A. palestinensis* [13], exhibited unique isoenzyme patterns for all the tested enzymes except MDH (Fig. 2, 3). However, it resembled *A. lenticulata* in its MDH isoenzyme patterns. Based on its trophic and cyst morphology and isoenzyme patterns, CDC: 1283:V013 was named *A. healyi* in honor of Dr. George R. Healy, Chief (retired), Protozoal Diseases Branch, Division of Parasitic Diseases, Centers for Disease Control.

The HK, ACP, Gd, MDH, and GPI enzyme patterns of CDC: 0884:V021 were very similar to those of *A. culbertsoni.* Though slight differences were noticed in the PGM, IDH, ALP, and LAP profiles of the two strains, the overall patterns of these enzymes were similar. However, the ME and ES isoenzyme patterns of CDC:0884:V021 were distinctly different from those of *A. culbertsoni.* Based on these observations, CDC:0884:V021 is being considered *A. culbertsoni* pending further studies.

Isoenzyme patterns of CDC-Fish-SK were similar to those of *A. royreba* for all the enzymes tested (not shown). CDC-Fish-SK was also morphologically very similar to *A. royreba.*

SDS-PAGGE of *Acanthamoeba* polypeptides. SDS-PAGGE and silver staining of proteins extracted from whole trophozoites revealed very complex profiles, with multiple major bands ranging from 14.4 to 200 kDa for each of the 13 strains examined (Fig. 4A). Despite the overall similarity noticed in the protein profiles of the 13 strains, members of group 1 could be easily distinguished from those of the other two groups. For example, members of groups 2 and 3 had a dense, dark staining band at about 44-46 kDa which was either absent or faint in group 1 organisms. Further, group 3 as well as *A. castellanii* had multiple dark staining bands in the region between 25 and 100 kDa that were either faint or not present in group 1 species. The most similar silver-stained protein profiles were those for *A. palestinensis-A. pustulosa,* those for *A. royreba* and CDC-FISH-SK in group 3 and those for *A. tubiashi–A. comandoni–A. echinulata* in group 1. *Acanthamoeba astronyxis* (group 1) and *A. castellani* (group 2) each presented remarkably unique profiles. A careful analysis of the silver-stained gel revealed that many of the strains possessed unique bands, e.g. *A. healyi* at 146.8 kDa; *A. culbertsoni* at 59 and 60.5 kDa; *A. palestinensis* at 94.9 and 31.3 kDa; *A. lenticulata* at 171.7 and 135.5 kDa; *A. royreba* at 165.1 and 70.9; *A. castellanii* at 130.2, 56.7 and a doublet at ~47 kDa; *A. astronyxs* at 120 and 87.7; *A. tubiashi* at 35.3 kDa; *A. comandoni* at 135.6 and 27.6 kDa; and *A. echinulata* at 54.5 kDa.

Immunoblot assays. Figure 4B–7 show the immunoblot patterns obtained when the separated *Acanthamoeba* polypeptides were reacted with rabbit antisera to *A. palestinensis* (Fig. 4B), *A. culbertsoni* (Fig. 5A), *A. royreba* (Fig. 5B), *A. healyi* n. sp. (Fig. 6A), *A. astronyxis* (Fig. 6B), *A. castellanii* (Fig. 7A), and *A. comandoni* (Fig. 7B). Hyperimmune rabbit antisera reacted extensively with the polypeptides of each of the 13 strains studied; however, the reactivity was most prominent in the homologous extracts. Major antigens were detected between 20 and 116.5 kDa. In these homologous reactions, the staining intensity was so great that some individual bands could not be easily distinguished in the photographs (Fig. 4–7). It is obvious from these studies that sera made against the group 3 species reacted extensively with the polypeptides of group 3 organisms, whereas they reacted moderately with those of *A. castellanii* (lane 8) and very little with the polypeptides of group 1 species. Only *A. astronyxis* (lane 9), among the group 1 species, appeared to react moderately with the group 3 and group 2 antisera. Conversely, sera made against group 1 species reacted prominantly with the antigens of group 1 organisms and sparsely with those of group 2 and group 3 organisms. All four species of group 1 produced a characteristic dense, dark staining band at about 30 kDa when reacted with the anti-*A. astronyxis* and anti-*A. comandoni* sera. Anti-*A. castellanii* serum reacted much more

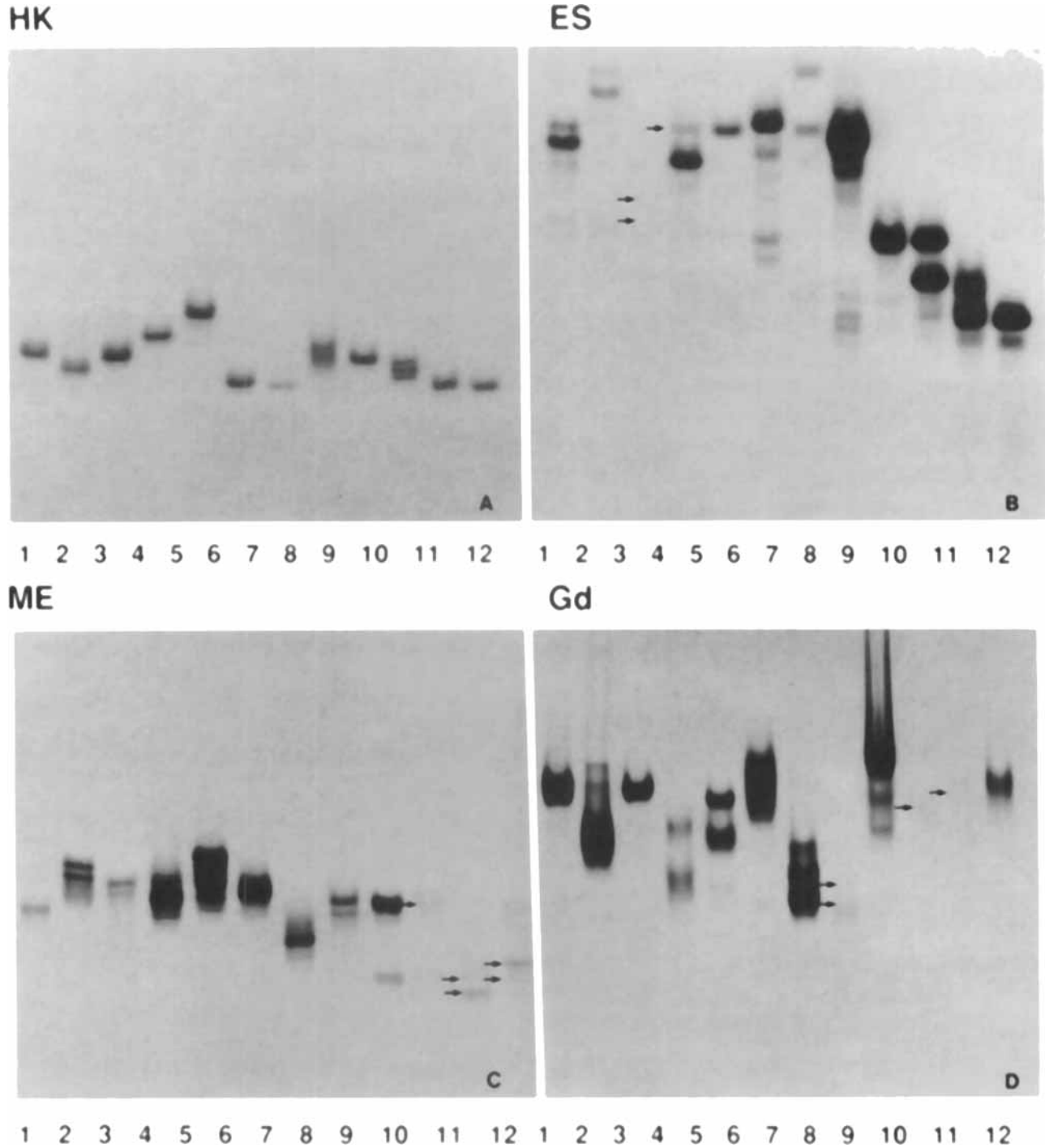

Fig. 2. Zymograms (by PAGGE) of *Acanthamoeba* spp. The numbers on the horizontal axis indicate species or strain numbers listed in Table 1. Arrows denote bleached bands. **A**. Hexokinase (HK). **B**. Esterase (ES). **C**. Malic enzyme (ME). **D**. Glucose-6-phosphate dehydrogenase (Gd).

strongly with the antigens of group 3 than with those of group 1 indicating closer affinities of this species with those of group 3 species.

DISCUSSION

Acanthamoeba spp. cause granulomatous amebic encephalitis (GAE) in humans and animals [9, 22]. In recent years, an increasing number of cases of GAE, especially in patients with AIDS, have been reported to the Centers for Disease Control [22]. As a result, etiologic agents are being isolated with increasing frequency. Since it is not always possible to identify the infective agent to the species level by morphologic criteria alone, other nonmorphologic criteria need to be used. Isoenzyme electrophoresis is one such nonmorphologic trait that has been used by others [3, 6, 25]. We have found that, for native proteins, high-resolution PAGGE appears to be a useful technique for

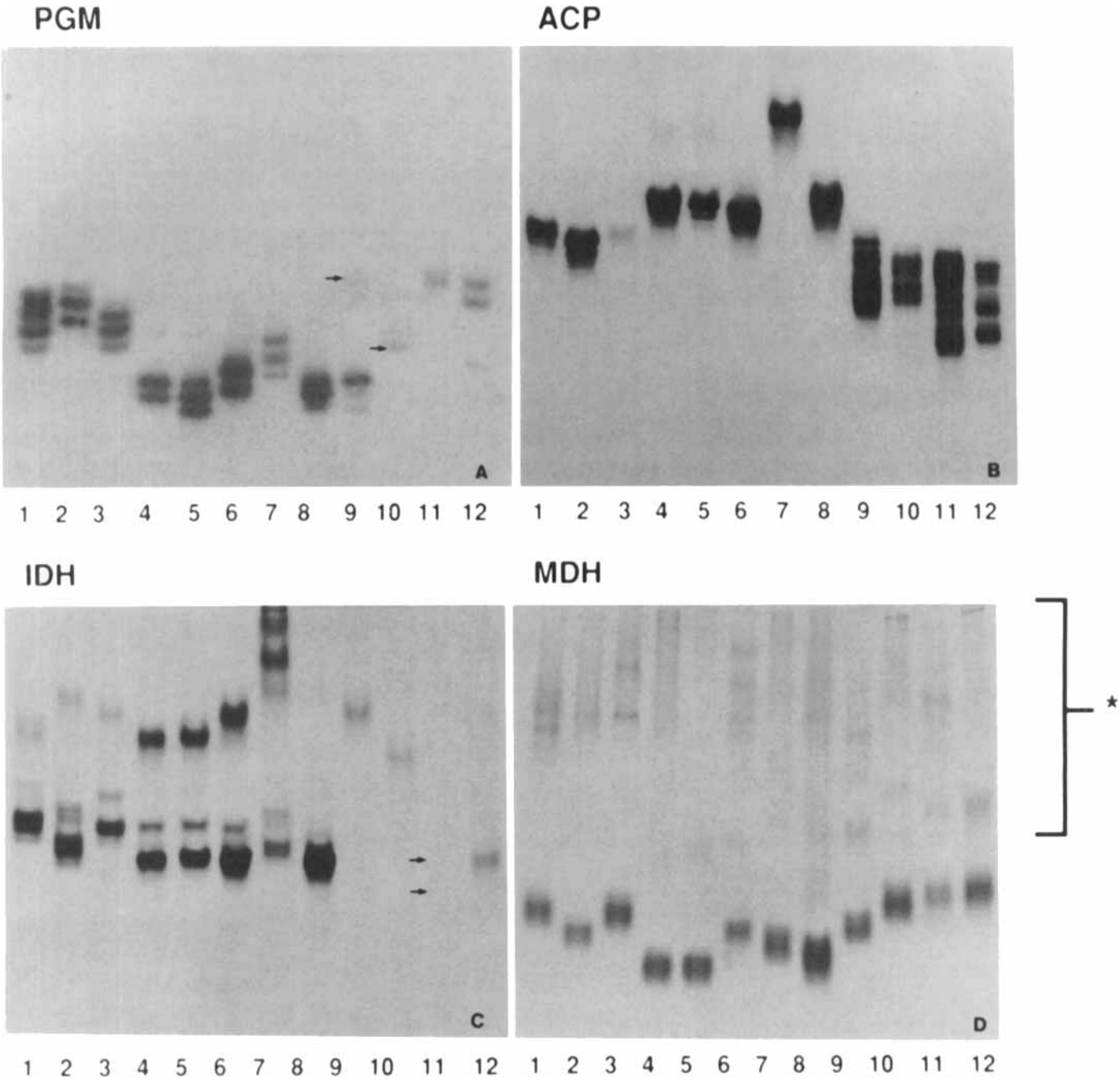

Fig. 3. Zymograms (by PAGGE) of *Acanthamoeba* spp. The numbers on the horizontal axis indicate species or strain numbers listed in Table 1. Arrows denote bleached bands. Asterisk on the bracket denotes nonspecific bands for MDH. **A**. Phosphoglucomutase (PGM). **B**. Acid phosphatase (ACP). **C**. Isocitrate dehydrogenase (IDH). **D**. Malate dehydrogenase (MDH).

the resolution of complex mixtures of polypeptides [11, 12]. Data we obtained using this technique confirm the results obtained by previous morphologic [14, 15], isoenzyme [6], and immunologic studies [24, 25] and add more information on the molecular characterization of *Acanthamoeba* belonging to groups 1 and 3 as defined by Pussard & Pons [15]. The differences we observed in the separated proteins strongly support the existence of the three morphologic groups, as reported before [7, 15, 19]. Further, the immunoblot profiles clearly indicate that group 1 species share only a few antigens with those of group 2 and group 3 species and hence are distinctly different from them. However, *A. castellanii,* a representative of group 2, shares a considerable number of antigens with those of group 3 species (Fig. 4B–6A) but few with those of group 1 species (Fig. 6A, 7B) indicating closer relationship with the group 3 strains rather than group 1 species.

An *Acanthamoeba* species (CDC:1283:V013) isolated from a brain biopsy specimen had been previously identified as *A. palestinensis* based on its morphologic characteristics and reactivity of the amebae in tissue sections with anti-*A. palestinensis*

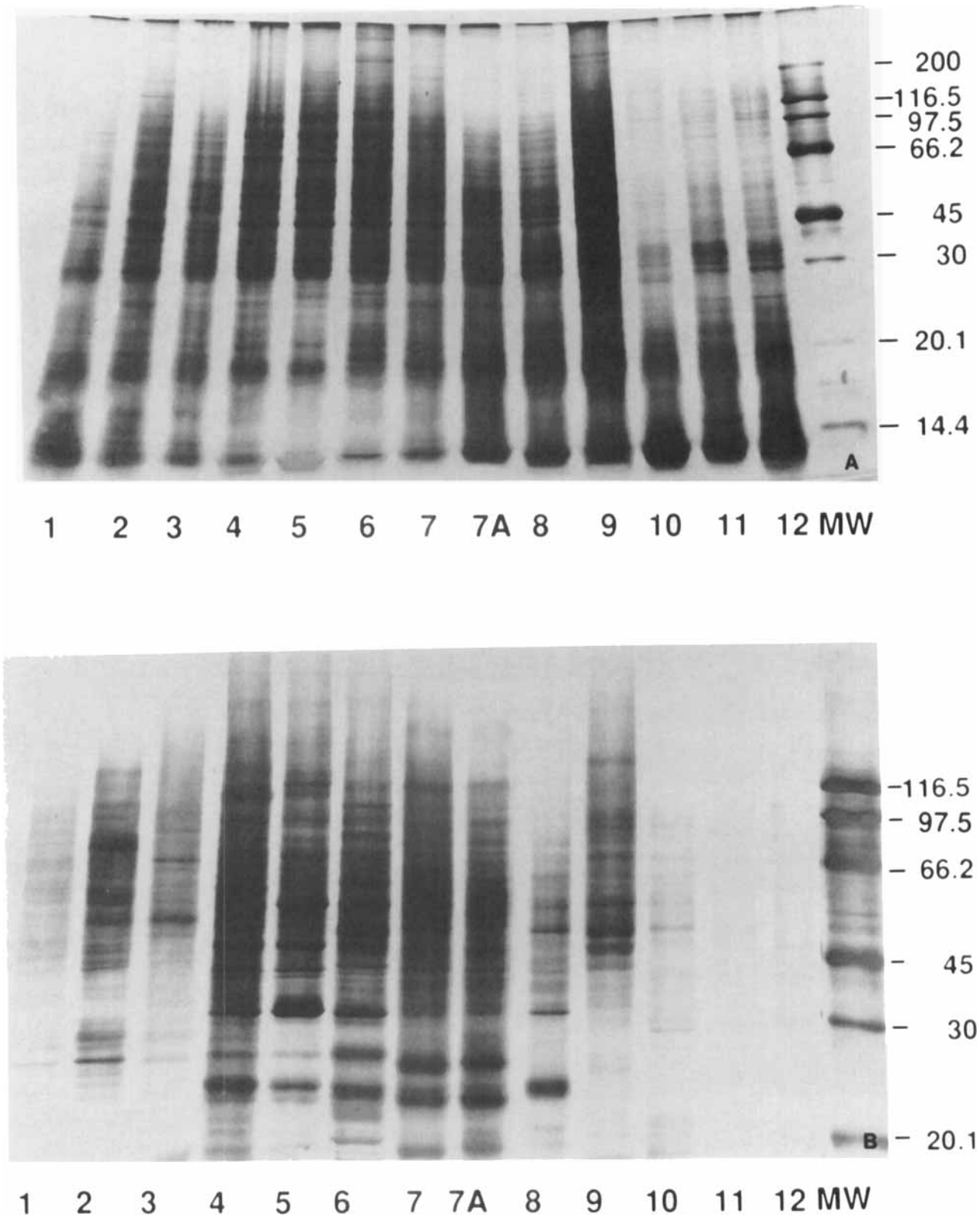

Fig. 4. Patterns (by SDS-PAGGE) of proteins of 13 *Acanthamoeba* strains. The numbers in the horizontal axis indicate species numbers listed in Table 1. Molecular mass markers (MW) are high (Bio-Rad) and low (Pharmacia). **A**. Silver stained gel. **B**. Immunoblot. The proteins were transferred to Immobilon membranes and treated with a 1:1,000 dilution of rabbit anti-*A. palestinensis*.

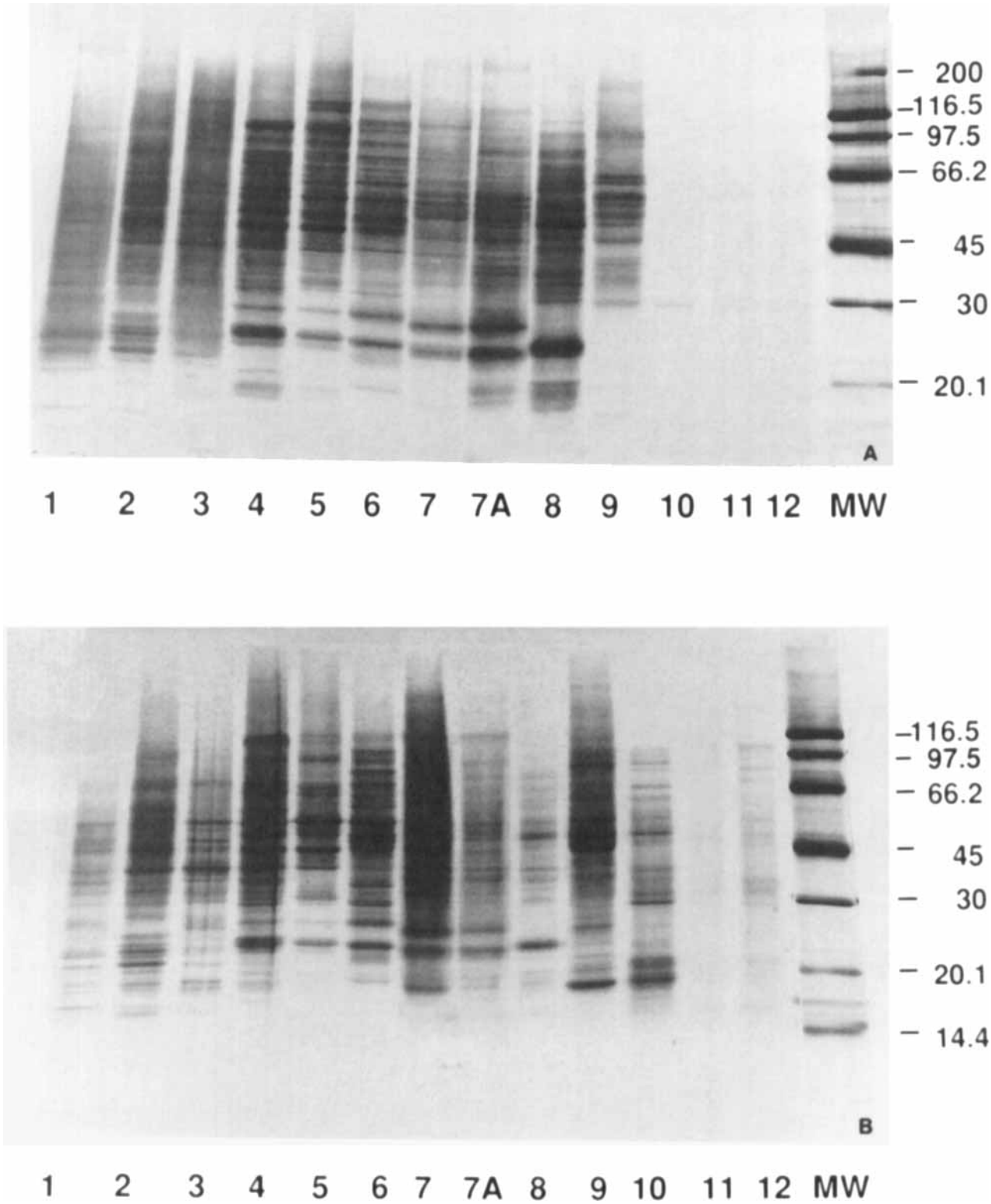

Fig. 5. Immunoblots of SDS-treated PAGGE separated proteins of 13 *Acanthamoeba* strains. Numbers in the horizontal axis and molecular mass markers (MW) are the same as those in Fig. 4. The proteins were transferred to Immobilon membranes and treated with a 1:1,000 dilution of **A**) rabbit anti-*A. culbertsoni* and **B**) anti-*A. royreba* sera.

serum [13]. Our more detailed study involving morphologic, enzymatic, and antigenic parameters clearly indicates that although this strain bears some morphologic resemblance to *A. palestinensis,* it is nevertheless very different from *A. palestinensis* and from all other species of *Acanthamoeba.* Hence it has been designated *A. healyi,* a new species.

CDC:0884:V021, the human brain isolate that was previously identified as *A. culbertsoni* [23], has been confirmed as *A. cul-*

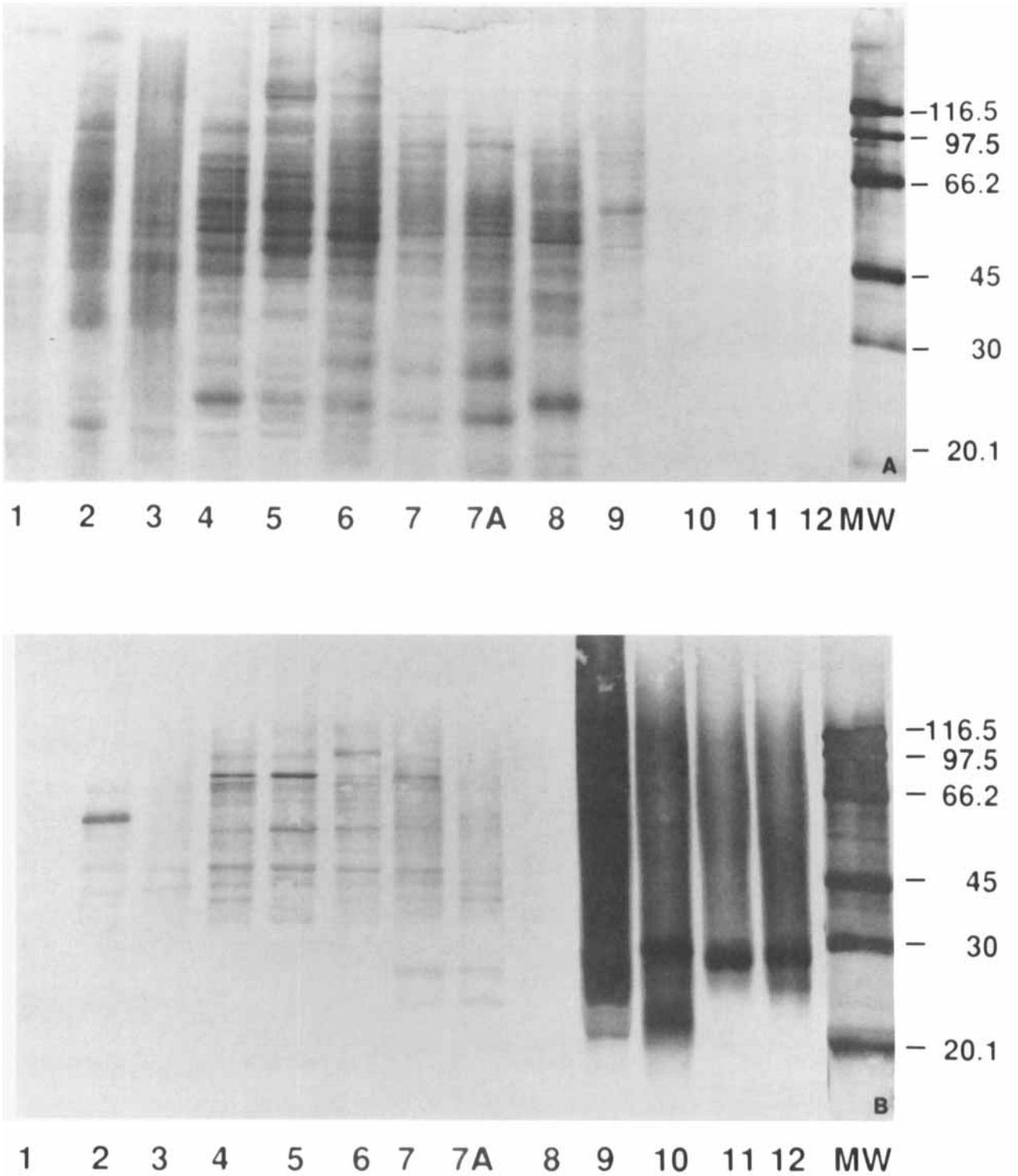

Fig. 6. Immunoblot profiles of SDS-treated PAGGE separated proteins of 13 *Acanthamoeba* strains. Numbers in the horizontal axis and molecular mass markers (MW) are the same as those in Fig. 4. The proteins were transferred to Immobilon membranes and treated with a 1:1,000 dilution of **A**) rabbit anti-CDC:1283:V013 (*A. healyi* n. sp.) and **B**) anti-*A. astronyxis* sera.

bertsoni because of its morphologic, enzymic, and antigenic similarity to the Lilly A-1 strain of *A. culbertsoni.*

We believe this is the first report of the isoenzyme make-up and antigenic characteristics of *A. tubiashi* which had not been cultivated axenically before. Our studies confirm the uniqueness of *A. tubiashi,* not only at the morphologic level, as noted previously [8], but also with regard to its isoenzyme patterns and antigenic profile. This is also the first time that axenically cultivated *A. echinulata* has been used to examine its isoenzyme and antigenic profiles. By unequivocally demonstrating the unique isoenzyme and protein profiles of *A. echinulata* and clearly showing its differences from those of *A. comandoni,* our results support the existence of four species in group 1 rather than three as reported previously [3, 6].

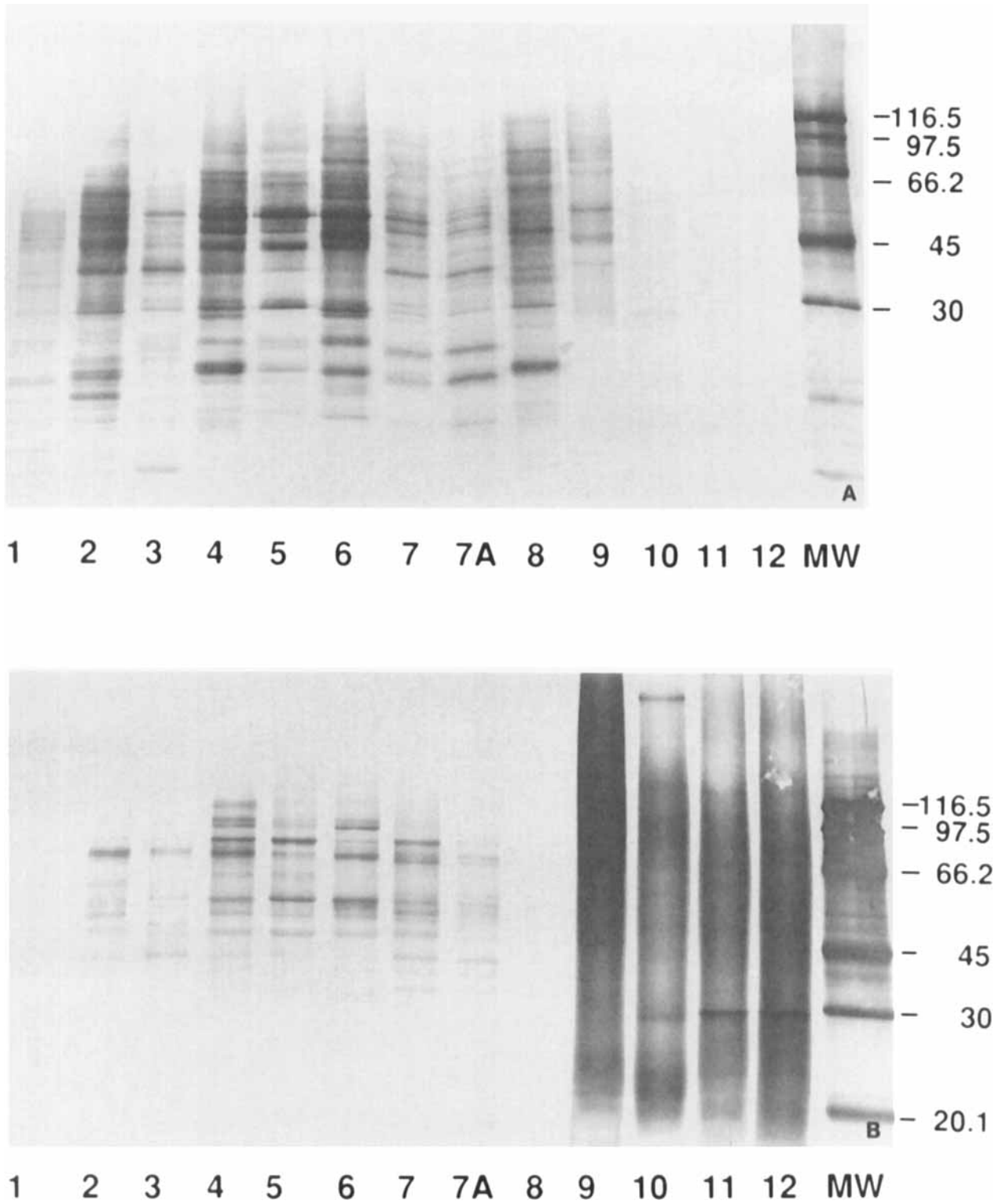

Fig. 7. Immunoblot profiles of SDS-treated PAGGE separated proteins of 13 *Acanthamoeba* strains. Numbers in the horizontal axis and molecular mass markers (MW) are the same as those in Fig. 4. The proteins were transferred to Immobilon membranes and treated with a 1:1,000 dilution of **A**) rabbit anti-*A. castellanii* and **B**) *A. comandoni* sera.

In group 3, *A. culbertsoni, A. palestinensis, A. lenticulata,* and *A. royreba* have been well characterized on the basis of morphologic [14, 15], isoenzyme [3, 6, 7], and immunoprecipitation [24] studies. However, *A. pustulosa* has been identified as an invalid synonym of *A. palestinensis,* based on their lack of pathogenicity to mice, morphologic similarity and identity of bands obtained for LAP, MDH, GPI, PGM, PE, and ADH [7, 14]. However, in our studies, *A. pustulosa* and *A. palestinensis* could be easily distinguished based on their HK, ES, PGM, ACP, Gd, and ME profiles even though they had similar MDH, ADH, IDH, and GPI profiles. Costas and Griffiths [3] also differentiated the two based on their ES and ACP profiles. Further, in

reactions with the anti *A. palestinensis* serum in the immunoblot assay (Fig. 4B) distinct differences were noted in the antigenic pattern of *A. pustulosa* from that of *A. palestinensis*. We therefore believe that *A. pustulosa* is a valid species.

A careful review of the isozyme data on groups 1 and 3 *Acanthamoeba* suggests that members of these groups can easily be differentiated from one another based only on HK, ACP, and ES profiles, which will reduce the investigator's time, effort, materials, and expense. If, however, numerical analysis of data is planned or other strains are isolated in the future that cannot be differentiated by these three enzymes, then other enzymes such as PGM, ME, and Gd may be used.

Profiles of SDS-treated and silver-stained polypeptides of *Acanthamoeba* species belonging to group 1 and to group 3 exhibited great within-group similarity, as evidenced by a large number of comigrating bands. Nevertheless, unique bands detected for each species allowed them to be differentiated, even though the unique bands were neither numerous nor prominent. Our results confirm the differences reported previously in the protein profiles of *Acanthamoeba* using agarose isoelectric focusing [6].

When probed with seven different polyclonal rabbit sera, immobilon membranes containing the electrotransfer peptides also confirmed the antigenic similarity among the species, as has been reported before for six different species of *Acanthamoeba* [25] based on immunoelectrophoresis patterns. However, we were able to recognize unique bands for each strain examined by probing with different antisera.

With PAGGE, a powerful technique for the separation of enzymes and antigens, the bands resolved are sharp and clear and thus facilitate the comparison of strains. In conclusion, we have used this technique to resolve the unique isoenzyme and antigenic patterns of two strains of *Acanthamoeba* isolated from human brain tissues and classify one as *A. healyi* n. sp. and the other as *A. culbertsoni*.

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