The Use of Physiological Characterizations in the Classification of Five Species of *Acanthamoeba*

By ALAN J. GRIFFITHS, LESLEY CURNICK, MARK D. UNITT
AND SUSAN L. WILCOX

Department of Microbiology, University College, Newport Road, Cardiff CF1 1XP

(Received 5 April 1978)

Physiological tests were devised and used to compare 15 strains representing five species of soil and freshwater amoebae assigned to the genus *Acanthamoeba*. The tests gave an acceptable level of reliability and the pattern of responses was not affected by the differing growth rates of the organisms. There was some degree of overlap between the strains, as shown by the high level of inter-species similarities in relation to those between strains of the same species. Previous classifications of *Acanthamoeba*, which are based solely on morphological criteria, do not adequately reflect the diversity of these amoebae.

INTRODUCTION

Small amoebae, variously assigned to the genera *Acanthamoeba*, *Hartmannella* and *Mayorella*, occur widely in soil and aquatic habitats and are probably some of the most common protozoa (Page, 1967, 1976). Some cause certain pathological conditions (Culbertson, 1971). As they are one of the few groups of protozoa which can be isolated and grown in axenic culture they are also being used increasingly in fundamental studies of cell physiology and biochemistry. However, their ecological relationships are poorly understood, due, in part, to the confusion of some of the existing classifications of the group which are based entirely on morphological criteria.

At present the most important taxonomic characters are: pseudopodial form; type of amoeboid movement; occurrence of a flagellate stage in the life-cycle; nuclear structure; cyst morphology and method of excystation. The use of such characters can be criticized on several grounds, for example, descriptions of pseudopodial form are often imprecise and cyst morphology can be extremely variable or modified significantly by the environment (Pussard, 1966; Stratford & Griffiths, 1978).

The DNA base compositions of representative strains range from 50 to 62% GC (Adam & Blewett, 1974) suggesting that existing classifications may not adequately reflect the diversity of this group of organisms. Physiological characters could be used more extensively (Adam, 1964*b*), and in this present study, methods have been devised to examine further the physiological diversity of 15 strains of amoebae now included in the genus *Acanthamoeba*.

METHODS

Strains and their routine maintenance. Fifteen strains representing five species of *Acanthamoeba* were examined (Table 1). All cultures were axenic and, after receipt, were maintained in PGY medium [0.75% (w/v) peptone, 0.75% (w/v) yeast extract, 1.5% (w/v) glucose; Difco or Oxoid peptones and yeast extracts could be used in this medium with equal effect]. Stock cultures (5 ml) were kept at laboratory temperature in small, screw-capped bottles. These were inoculated with 0.5 ml of a culture which had been maintained similarly and subcultures were made every 4 to 6 weeks.

Tests used to characterize cultures of trophozoites. Tests devised for this study are listed in Table 2. PGY

Table 1. *Designations and origins of Acanthamoeba cultures*

From the Culture Centre of Algae and Protozoa, Storey's Way, Cambridge

<i>A. castellanii</i>	*1501/1a, 1501/1b, 1501/2b
<i>A. polyphaga</i>	1501/3a, 1501/3b, 1501/3g, 1501/3h
<i>A. rhyodes</i>	1534/3
<i>A. astronyxis</i>	1534/1
<i>A. palestinensis</i>	1547/1

From Mrs K. M. G. Adam, Zoology Department, Edinburgh University

<i>A. castellanii</i>	Neff strain, Harrison strain
<i>A. polyphaga</i>	Page isolate P23
<i>A. rhyodes</i>	Singh strain
<i>A. palestinensis</i>	Reich strain

* Culture Centre catalogue numbers.

Table 2. *Tests used in the comparison of Acanthamoeba cultures*

No.	Test	No.	Test
1	Acanthapodia present in amoebae	30	0.25% NaNO ₃ in 5 d
	Growth at:	31	0.25% NaNO ₃ in > 5 d
2	37 °C	32	0.25% (NH ₄) ₂ SO ₄ in 6 d
3	30 °C in 5 d	33	0.25% (NH ₄) ₂ SO ₄ in > 6 d
4	30 °C in > 5 d	34	0.25% NaNO ₂ in 10 d
5	25 °C in 5 d	35	0.25% NaNO ₂ in > 10 d
6	25 °C in > 5 d	36	50 µg chloroquine ml ⁻¹ in 10 d
7	15 °C in 5 d	37	50 µg chloroquine ml ⁻¹ in > 10 d
8	15 °C in > 5 d	38	25 µg sodium arsenite ml ⁻¹
9	6 °C	39	1 mg chloramphenicol ml ⁻¹
10	pH 3	40	0.5 µg cycloheximide ml ⁻¹ in 4 d
11	pH 4 in 5 d	41	0.5 µg cycloheximide ml ⁻¹ in > 4 d
12	pH 4 in > 5 d		Growth in:*
13	pH 5 in 6 d	42	1% malt extract
14	pH 5 in > 6 d	43	5% malt extract
15	pH 7 in 5 d	44	DM2
16	pH 7 in > 5 d	45	DM3
17	pH 9	46	4% Mycological Peptone
18	Growth in deep tubes	47	CVG
	Growth in the presence of:	48	PY
19	1.0% bile salts	49	1/3-strength PGY in 5 d
20	2.0% NaCl	50	1/3-strength PGY in > 5 d
21	1.5% NaCl	51	1/3-strength PGY in 15 d
22	1.0% NaCl in 10 d	52	1/3-strength PGY in > 15 d
23	1.0% NaCl in > 10 d	53	BHI in 5 d
24	1.0% urea	54	BHI in > 5 d
25	0.6% urea	55	Amoebae encyst in PGY/Mg
26	0.4% urea in 7 d	56	Cysts germinate after desiccation
27	0.4% urea in > 7 d	57	Cysts resistant to freezing at -25 °C for 24 h
28	0.1% urea in 7 d	58	Cysts resistant to 1 M-HCl for 24 h
29	0.1% urea in > 7 d	59	Cysts survive at 60 °C for 10 min
		60	Cellulose present in cell wall

In all tests, except nos 42 to 54, growth was assessed in PGY medium with or without supplements. Growth was scored as present or absent after 21 d unless otherwise stated. Concentrations expressed as % were all on a w/v basis.

* DM2 and DM3 were the defined media of Adam (1964*a, b*); CVG medium contained 0.75% (w/v) Difco vitamin-free Casamino acids, 1.5% (w/v) glucose and a vitamin mixture (Adam, 1959, 1964*a*); PY medium was the PGY medium without glucose; BHI was Difco Brain Heart Infusion.

was the basal medium for all tests except nos 42 to 54. Supplements to PGY were added before autoclaving to give the final concentrations shown for tests nos 19 to 41. The pH values of the media were adjusted with 1 M-HCl or 1 M-NaOH. Media were sterilized by autoclaving at 121 °C for 15 min, with the exception of the defined media which were sterilized as described by Adam (1959, 1964a). All the tests, except no. 18, were done in 50 ml conical flasks containing 5 ml of the appropriate medium. Growth in deep tubes (test no. 18) was tested in 15 ml PGY dispensed in test tubes and covered with 1 cm of liquid paraffin. Cultures were inoculated with 1 ml of a mid-exponential phase culture which had been prepared by incubating the cells in PGY in a shaking water-bath for 2 to 3 d at 30 °C. Test cultures were incubated at 30 °C without shaking and examined for growth at daily intervals for 21 d. In most tests, growth was indicated by development of turbidity and confirmed by microscopic examination for the presence of trophozoites. Haemocytometer counts were used only for those tests which did not give strong responses (nos 42 to 47).

Tests used for cysts. Cysts were obtained by adding MgCl_2 (final concentration 50 mM) to cultures which had been grown in PGY for 5 d at 30 °C in a shaking water-bath (Chagla & Griffiths, 1974). After further incubation for 2 d, 5 ml portions were centrifuged aseptically at 500 g for 5 min. Cyst pellets were washed with fresh, sterile 50 mM- MgCl_2 solution and centrifuged again. Washed cyst pellets were tested aseptically (i) for their ability to withstand desiccation (cysts were stored over silica gel until a constant weight was obtained); (ii) for their ability to survive freezing at -25 °C for 24 h; (iii) for their resistance to 1 M-HCl (cysts were suspended in the acid for 24 h then recovered by centrifuging and washed with sterile water until the washings were neutral); and (iv) for their ability to survive heating at 60 °C for 10 min (cysts were heated in 5 ml sterile water and recovered by centrifuging). To assess survival in each case, cysts were resuspended in fresh PGY, incubated without shaking at 30 °C and examined for growth at daily intervals for 21 d. The presence of cellulose in the cyst walls was determined by placing a few drops of Schulze's zinc-chloriodide reagent (E. Gurr, London SW14) on a heat-fixed smear of the cells on a microscope slide; a purple colouration of the cyst showed the presence of cellulose.

Scoring and analysis of results. Results were recorded as positive or negative and the 17 tests which were based on a differentiated growth response time were coded additively. Similarities were estimated using the simple matching coefficient (Sokal & Michener, 1958). To test the possibility that the similarities between the strains could be affected by differences in growth rates, the results were also re-calculated to take into account any differences in Vigour and Pattern (Sneath, 1968).

RESULTS AND DISCUSSION

Evaluation of tests

None of the tests (Table 2) were negative for all the strains and only three were wholly positive (growth in deep tubes, resistance to chloramphenicol and growth in PY medium). The responses of the strains ranged from 86% of the tests positive for *A. castellanii* 2b to 58% positive for *A. castellanii* 1a.

Tests were duplicated with three strains (*A. castellanii* Neff, *A. castellanii* 1a and *A. rhysodes* Singh) on separate occasions and using different batches of medium. Similarities of 95 to 100% were found between the duplicated responses of each strain, suggesting a high consistency of test results.

As the tests relied on the presence or absence of growth, strains might have exhibited a low level of similarity because of their different growth rates: generation times ranged from 13.5 h (*A. castellanii* Neff) to 74.0 h (*A. astronyxis*). However, this slowest growing strain had a similarity of 80% with *A. polyphaga* P23 which had a generation time of 20.0 h, and *A. castellanii* Neff had a similarity of 78% with *A. castellanii* Harrison (generation time 44.0 h). Re-calculation of the similarities to take into account Vigour and Pattern differences (Sneath, 1968) had no appreciable effect on the results of the comparisons between any of the strains. It seems unlikely, therefore, that differing growth rates had any significant effect on the patterns of results.

Three strains of *A. castellanii* (1a, 1b and Neff) were originally derived from the same isolate, and two strains of *A. polyphaga* (P23 and 3a) were originally derived from the same clone but were separated 8 to 9 years ago. The three strains of *A. castellanii* had an average similarity of 66.3% in their responses to the physiological tests and those of *A. polyphaga* had a similarity of only 62% to each other. These results may reflect some lack of

Table 3. *Percentage similarities* between and within species of Acanthamoeba*

	<i>A. castellanii</i>	<i>A. polyphaga</i>	<i>A. rhysodes</i>	<i>A. palestinensis</i>	<i>A. astronyxis</i>
<i>A. castellanii</i>	70.9 ± 12.3				
<i>A. polyphaga</i>	68.6 ± 10.5	68.0 ± 11.5			
<i>A. rhysodes</i>	66.1 ± 7.8	63.1 ± 5.2	73.0†		
<i>A. palestinensis</i>	67.6 ± 6.3	64.8 ± 5.7	74.5 ± 1.9	78.0†	
<i>A. astronyxis</i>	64.4 ± 7.8	70.6 ± 7.2	77.5 ± 3.5	71.5 ± 9.2	100‡

* Mean percentage similarities are shown, together with standard deviations where applicable (†, only two strains examined; ‡, one strain examined).

Table 4. *Tests which gave the best differentiation of Acanthamoeba species (numbers of positive responses)*

Test	<i>A. castellanii</i>	<i>A. polyphaga</i>	<i>A. rhysodes</i>	<i>A. palestinensis</i>	<i>A. astronyxis</i>
No. of strains tested	5	5	2	2	1
Growth at:					
pH 4 (5 d)	4	2	1	0	1
pH 7 (5 d)	4	4	1	1	0
Growth in:					
BHI (5 d)	4	0	2	2	0
‡ PGY (5 d)	4	3	2	1	0
DM2 (21 d)	4	3	2	2	0
Growth in the presence of:					
1.0% NaCl (10 d)	4	3	0	1	0
0.6% urea (21 d)	4	4	0	1	0
NaNO ₃ (5 d)	4	4	1	0	1
Cycloheximide (4 d)	5	3	2	0	0
Chloroquine (10 d)	4	4	0	0	1
Arsenite (21 d)	1	1	0	2	0

consistency in the tests but it is, perhaps, more likely that they reveal a physiological variability between the strains resulting from the selection of variants by the different maintenance conditions and inoculation regimes to which they have been subjected since their separation.

Comparisons within and between species

There was a relatively low level of overall similarity (69.9%) between the 15 strains of *Acanthamoeba* but a considerable degree of overlap between the species (Table 3). For example, the similarity between strains of *A. castellanii* and *A. polyphaga* was higher than that between strains of *A. polyphaga* alone and the strains of *A. rhysodes* showed a higher average similarity to those of *A. palestinensis* than they did to each other. Consequently, even the tests which gave the best differentiation between the species do not allow an unequivocal separation to be made on physiological grounds (Table 4). Such physiological variability is also found in other groups of microbes, and need not be an obstacle to the use of physiological characterizations for the identification of the various species of *Acanthamoeba*.

Willaert (1976) has suggested that *A. castellanii*, *A. polyphaga* and *A. rhysodes* are a homogeneous group on the basis of their antigenic relationships but that *A. palestinensis* and *A. astronyxis* could both be considered as distinct from *A. castellanii*. Thus, on the evidence of physiological and antigenic comparisons there would seem to be some support for a re-examination of the taxonomy of *Acanthamoeba*. The small number of strains which were available to us to carry out this investigation were clearly insufficient to form the basis of a thorough taximetric study and the accumulation of a representative collection of cloned isolates is now one of the most urgent priorities for any future work along these

lines. The question of test reliability must also continue to receive attention and, although the use of cloned cultures will be of great benefit, the conditions under which the tests are conducted and the cultures maintained must be rigorously standardized. The tests used in this study were those which, after preliminary investigations, gave the best reproducibility. There are, however, other biochemical and physiological features which could be incorporated into future work. Certain of the chemical criteria which have been used with some success in taxonomic studies of other groups of microbes could be developed for use with the small soil and freshwater amoebae. The lipid and sterol composition of some strains of axenic amoebae (Halevy & Finkelstein, 1965; Halevy *et al.*, 1966) and the amino-acid composition of species of *Amoeba* and *Chaos* (Friz, 1974) have already been investigated. As the amoebae are essentially phagotrophic and incorporate growth medium directly into intracellular vacuoles some caution will, however, be necessary in studies of this nature.

The methods described in this paper are mainly applicable to axenic cultures of amoebae. Ecological investigations would undoubtedly be facilitated by the development of physiological tests which could be used with monoxenic cultures. Drug resistance and tolerance to a range of temperatures and salinities would seem to offer some of the more promising starting points for future work along these lines.

REFERENCES

- ADAM, K. M. G. (1959). The growth of *Acanthamoeba* sp. in a chemically defined medium. *Journal of General Microbiology* **21**, 519–529.
- ADAM, K. M. G. (1964*a*). The amino-acid requirements of *Acanthamoeba* sp. Neff. *Journal of Protozoology* **11**, 98–100.
- ADAM, K. M. G. (1964*b*). A comparative study of hartmannellid amoebae. *Journal of Protozoology* **11**, 423–430.
- ADAM, K. M. G. & BLEWETT, D. A. (1974). Studies on the DNA of *Acanthamoeba*. *Annales de la Société belge de médecine tropicale* **54**, 387–393.
- CHAGLA, A. H. & GRIFFITHS, A. J. (1974). Growth and encystation of *Acanthamoeba castellanii*. *Journal of General Microbiology* **85**, 139–145.
- FRIZ, C. T. (1974). Taxonomic comparisons of *Amoeba proteus*, *Amoeba dubia* and *Chaos carolinensis*. I. Observations using the principles of numerical taxonomy. *Archiv für Protistenkunde* **116**, 236–243.
- HALEVY, S. & FINKELSTEIN, S. (1965). Lipid composition of soil amoebae. *Journal of Protozoology* **12**, 250–253.
- HALEVY, S., AVIVI, L. & KATAN, H. (1966). Sterols of soil amoebas and *Ochromonas danica*. Phylogenetic approach. *Journal of Protozoology* **13**, 480–483.
- PAGE, F. C. (1967). Re-definition of the genus *Acanthamoeba* with descriptions of three species. *Journal of Protozoology* **14**, 709–724.
- PAGE, F. C. (1976). *An Illustrated Key to Freshwater and Soil Amoebae*. Scientific Publication 34. Ambleside: Freshwater Biological Association.
- PUSSARD, M. (1966). Le genre *Acanthamoeba* Volkonsky 1931 (Hartmannellidae: Amoebida). *Protistologica* **2**, 71–93.
- SNEATH, P. H. A. (1968). Vigour and pattern in taxonomy. *Journal of General Microbiology* **54**, 1–11.
- SOKAL, R. R. & MICHENER, C. D. (1958). A statistical method for evaluating systematic relationships. *Kansas University Science Bulletin* **38**, 1409–1438.
- STRATFORD, M. P. & GRIFFITHS, A. J. (1978). Variations in the properties and morphology of cysts of *Acanthamoeba castellanii*. *Journal of General Microbiology* (in the Press).
- WILLAERT, E. (1976). Étude immuno-taxonomique des genres *Naegleria* et *Acanthamoeba* (Protozoa: Amoebida). *Acta zoologica et pathologica antverpiensis* **65**, 4–239.