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ORIGINAL PAPER

Acanthamoeba strains show reduced temperature tolerance after long-term axenic culture

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Abstract *Acanthamoeba* is a genus of free-living organisms that can be found in various habitats. We investigated the physiological characteristics of 15 *Acanthamoeba* isolates, representing five genotypes (T4, T5, T6, T7, and T11) of both clinical and nonclinical origins. Moreover, in order to evaluate possible alterations from long-term culture, old and fresh isolates were included, and results were compared to a previous study. We found that there is no significant difference in physiological characteristics between genotypes. However, *Acanthamoeba* strains that had been grown in axenic culture over long periods of time adapted to axenic growth. Overall growth rates under-agarose migration and particularly, temperature tolerance decrease after long-term axenic culture at room temperature. The only trait that remained rather constant was the cytopathic effect.

Introduction

Acanthamoebae are free-living organisms that occur in various types of environments, including water and soil (Boost et al. 2008; Thomas et al. 2008). Under certain conditions, *Acanthamoeba* can cause human infections such as *Acanthamoeba* keratitis (AK), an acute sight-threatening corneal infection often related to contact lens misuse (Illingworth and Cook 1998) and granulomatous amoebic encephalitis (GAE), a severe and usually fatal chronic brain infection that occurs mostly in immunocompromised individuals (Martinez and Visvesvara 1997; Walochnik et al. 2008). The pathogenesis of *Acanthamoeba* is not entirely understood and the exact factors determining the pathogenic potential of a particular strain are unclear. By morphology-based classification, *Acanthamoeba* has been divided into three major morphological groups (Pussard and Pons 1977). While representatives of group I seem to be nonpathogenic, the majority of clinical isolates belongs to group II. Particularly, GAE-causing strains can also be found in group III. However, both group II and group III also contain clearly nonpathogenic strains. *Acanthamoeba* has been classified into 15 genotypes by 18S rRNA gene sequencing (Gast et al. 1996; Stothard et al. 1998; Horn et al. 1999; Gast 2001; Hewett et al. 2003). Most often associated with diseases is genotype T4, however, the majority of proven nonpathogenic isolates also belongs to this genotype (Stothard et al. 1998; Booton et al. 2004; Yera et al. 2008). Thus, also genotyping does not enable discrimination between pathogenic and nonpathogenic strains.

Physiological characteristics such as temperature tolerance (Griffin 1972), growth in defined media (De Jonckheere 1977), the ability to migrate in an "under-agarose system" (Thong and Ferrante 1986), and the cytopathic effects on human cell lines (Cursons and Brown 1978; De Jonckheere 1980) have been found to be pathogenicity related. However, it has not yet been clarified whether these are indeed constant characters. Adaptations to prolonged axenic culture, such as changes in cellular enzyme activity and alterations in drug sensitivity (Stevens and O'Dell 1974; Kasprzak et al. 1985), attenuation of virulence (Mazur and Hadas 1994), and losses in the ability to encyst (Koehsler et al. 2008) have been reported. Therefore, it seems possible that long-term culture might also affect other physiological characteristics of *Acanthamoeba*, including those related to pathogenicity.

The aim of this study was to assess possible physiological alterations of *Acanthamoeba* strains after long-term

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axenic culture. Therefore, eight strains that had been investigated in detail 10 years ago (Walochnik et al. 2000b) and had been in axenic culture since then were screened for their growth rates, cytopathic effects, temperature tolerances, and under-agarose migrations. In order to also reveal possible genotype-specific traits, seven more strains from different genotypes and of both clinical and nonclinical origins were included in this study.

Materials and methods

Amoeba strains

A total of 15 *Acanthamoeba* isolates representing all three morphological groups and five genotypes from clinical and nonclinical origins were investigated. Detailed information on all investigated strains is given in Table 1. Briefly, strains 4CL (T4), 9GU (T4), 2HH (T4), 1BU (T4), 3ST (T4), 11DS (T6), and 4RE (T11) were isolated in our lab between 1997 and 1999, investigated in detail for the first time in 1999 (Walochnik et al. 2000a, b) and kept in axenic culture without interruption since then. Strain PAT06 (T4) was isolated in 2006 (Koehsler et al. 2008), and strains SPA08 (T4), DU08 (T4), 3250 (T4), and ZOO9 (T11) were just recently isolated in our lab. Strains 72/2 (T5) and Pb30/40 (T7) were kindly provided by Dr. Michel (Koblenz, Germany). Strain 72/2 had originally been isolated approximately 25 years ago from the nasal mucosa of a healthy individual but had proven to be highly virulent to mice after intranasal instillation (Michel et al. 1982; De Jonckheere and Michel 1988). The strain used in the current study is the reisolate from the brain of the mouse. Strain Pb30/40 was originally isolated from a greenhouse in Hamburg, Germany approximately 20 years ago. Both strains have been kept in axenic culture in our lab since 1997. Strain NEFF, isolated from soil about 50 years ago (Neff 1957), was purchased from ATCC.

Cultivation of amoebae

All strains were grown in sterile filtrated proteose peptone yeast extract-glucose medium (PYG) in 25-cm^2 tissue culture flasks (ASAI Glass, Osaka, Japan) at room temperature. For plate cultures, amoebae were grown on nonnutrient agar plates covered with a lawn of *Escherichia coli* (Walochnik et al. 2000b).

Cytopathic effects

Cytopathic effects of amoebae were evaluated using HEp-2 cells as described previously (Walochnik et al. 2000b). HEp-2 cells were cultured in a 1:1 mixture of PC-1 (Bio-Whittaker, Walkersville, MD, USA) and CO_2-independent medium (Life Technologies, Ltd., Paisley, Scotland) supplemented with L-glutamine (2 mM) in 75-cm^2 tissue culture flasks (ASAI Glass, Osaka, Japan) at 37°C under sterile conditions. Briefly, amoebae were counted with a hemacytometer and 1 ml of a suspension of each strain with 10^5 trophozoites per milliliter was inoculated onto a monolayer of HEp-2 cells. Cocultures of amoebae and cells were incubated at 34°C. Complete lysis of the monolayer within 48 h was scored three plus (+++), 50% lysis of the monolayer within 48 h was scored two plus (++), and 25% lysis of the monolayer within 48 h was scored one plus (+).

Table 1 Summary information on the investigated *Acanthamoeba* strains

Genotype	Strain	Group	Origin	Source or reference
Clinical isolates				
T4	1BU	II	Keratitis patient, cornea	Walochnik et al. 2000b
T4	2HH	II	Keratitis patient, cornea	Walochnik et al. 2000b
T4	3ST	II	Keratitis patient, cornea	Walochnik et al. 2000b
T4	PAT06	II	Keratitis patient, cornea	Koehsler et al. 2008
T4	DU08	II	Keratitis patient, cornea	New isolate
T4	SPA08	II	Keratitis patient, cornea	New isolate
T5	72/2	III	Mouse brain	Michel et al. 1982
T6	11DS	II	Keratitis patient, cornea	Walochnik et al. 2000a
T11	ZOO9	II	Anaconda tissue	New isolate
Nonclinical isolates				
T4	NEFF	II	Soil	Neff 1957
T4	3250	II	Water	New isolate
T4	4CL	II	Contact lens case	Walochnik et al. 2000b
T4	9GU	II	Contact lens case	Walochnik et al. 2000a
T7	Pb30/40	I	Greenhouse	Michel et al. 2004
T11	4RE	II	Contact lens case	Walochnik et al. 2000a

Growth curves in axenic culture and generation times

Amoebae were cultured with an initial cell density of 1×10^3 cell per milliliter in sterile PYG medium. The amoebae were counted every 24 h with a hemacytometer over a period of 8 days. Growth rates and generation times were calculated.

Growth rates on plates, temperature tolerances, and under-agarose migrations

The growth rates of the amoebae were evaluated on plates as previously described (Walochnik et al. 2000b). Amoebic cysts were harvested from plates and counted with a hemacytometer. Then 1 μl of 10^5 cysts per milliliter was inoculated onto the center of an agar plate coated with 100 μl of a 48-h-old culture of *E. coli*. Plates were marked with three concentric circles spaced at 15 mm intervals on the bottom side, incubated at 34°C, 37°C, and 40°C and checked daily under a phase-contrast microscope. Growth rates were recorded after 48 h and assessed according to the number of circles already traversed by the amoeba front. Under-agarose migration of the amoebae was recorded after 72 h and observed daily over a period of 1 week.

All experiments were carried out in triplicates. Means were calculated from all individual tests. In case of the plus scoring, the absolute means were brought to a round figure.

Results

Follow-up after long-term axenic culture

The most interesting observation was that while general growth rates only slightly decreased over time, ability to grow at high temperatures (37–40°C) seems to be markedly diminished after 10 years of axenic culture at 25–30°C (Table 2). None of the previously thermophilic strains still showed growth at 40°C. Also, the ability to migrate in an under-agarose system slightly decreased after long-term axenic culture. Interestingly, on the other hand, the strain that had shown very weak growth in axenic culture as well as on agar plates in the previous study, strain 4RE, now grew very well. Generally, all strains showed high growth rates at temperatures up to 34°C. Thus, strains seem to "adapt" to axenic culture and the concurrent culture conditions. The only trait that remained rather constant over the 10-year period was the cytopathic potential.

Table 2 Ten year follow-up of physiologic characteristics of different *Acanthamoeba* strains

Strain	Axenic culture	Cytopathic effect	Growth rate			Under-agarose migration
	RT	34°C	34°C	37°C	40°C	RT
1BU (T4)						
(a)	++	++	+++	++	−	+
(b)	+++	++	+++	+++	+++	++
2HH (T4)						
(a)	+	+++	+++	+++	−	++
(b)	+++	+++	+++	+++	+++	++
3ST (T4)						
(a)	+++	++	++	+	−	+
(b)	+++	++	+++	+++	+	++
4CL (T4)						
(a)	++	−	+++	++	−	+
(b)	++	+	+++	++	-	+
9GU (T4)						
(a)	++	++	+++	−	−	+
(b)	++	++	+++	++	−	+
4RE (T4)						
(a)	+++	−	++	++	−	−
(b)	+	-	+	+	−	−
72/2 (T5)						
(a)	++	+++	+++	+++	+++	++
(b)	+++	+++	+++	+++	+++	++
11DS (T6)						
(a)	+++	++	++	+	−	++
(b)	+++	+++	+++	+++	+++	+++

RT room temperature, (*a*) recent data, (*b*) data from 1999 (Walochnik et al. 2000b)

Growth rates in axenic culture

Overall generation times in axenic culture ranged from 12 to 38 h and based on their growth curves the isolates could be divided into three different groups, fast growing (generation time, <20 h), moderately growing (generation time, 20–30 h), and slow growing (generation time, >30 h) strains. Each group contained clinical and nonclinical as well as fresh and old isolates, and there was also no correlation between growth rates and genotypes.

Growth rates within genotype T4 are shown in Fig. 1. Interestingly, the highest growth rate was observed for strain 3ST (generation time, 12 h) and the lowest growth rate for strain 2HH (generation time, 37 h), both keratitis isolates, which had been cultured axenically for 10 years. Moderately growing strains were SPA08, PAT06, 1BU, and 4CL, and slowly growing strains were strains 2HH, NEFF, 9GU, DU08, and 3250.

Comparison of growth rates between different *Acanthamoeba* genotypes is shown in Fig. 2. The fastest growing strain again was strain 3ST (T4); the highest cell counts after 8 days were reached by strain 11DS (T6). However, there were major differences of growth rates within each genotype as already seen within T4. Examples are strains 4RE (generation time, 20 h) and ZOO9 (generation time, 34 h), both T11.

Altogether, growth rates did not correlate with either age, origin, or genotype.

Cytopathic effects, temperature tolerances, and under-agarose migrations

An overview of physiological capabilities of all strains according to origin and genotype is given in Table 3.

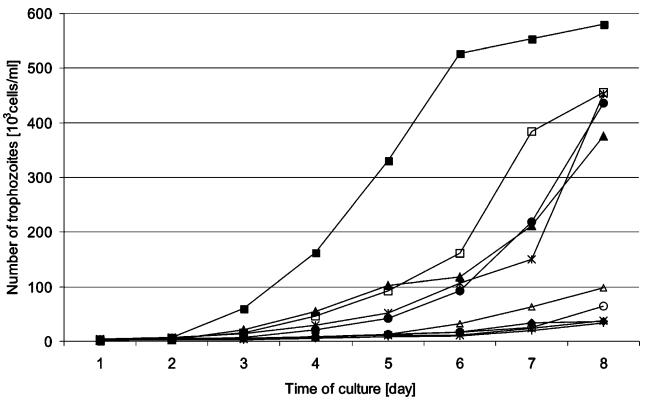

Fig. 1 Growth curves of *Acanthamoeba* genotype T4 in axenic culture at room temperature; strain 3ST (clinical; ■), strain SPA08 (clinical; □), strain PAT06 (clinical; ▲), strain 3250 (nonclinical; Δ), strain 4CL (nonclinical; ●), strain 9GU (nonclinical; ○), strain DU08 (clinical; ♦), strain 1BU (clinical; *), strain 2HH (clinical; ×), strain NEFF (nonclinical; +). Each *point* represents the mean number of trophozoites (calculated from triplicates). Deviations from the mean were generally *below* 3.50

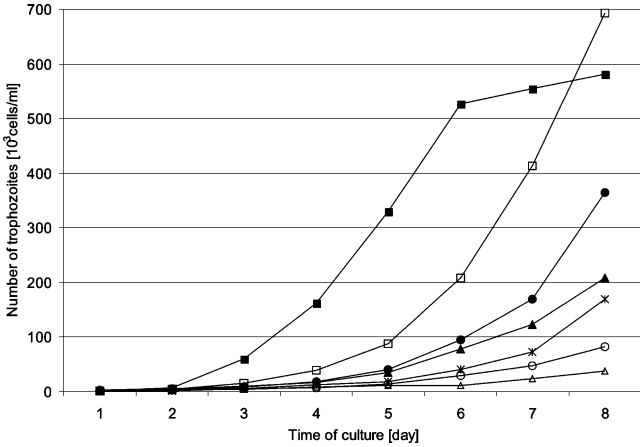

Fig. 2 Growth curves of different *Acanthamoeba* genotypes in axenic culture at room temperature; strain 2HH (T4) (Δ), strain 3ST (T4) (■), strain 72/2 (T5) (*), strain 11DS (T6) (□), strain Pb30/40 (T7) (▲), strain 4RE (T11) (●), and strain ZOO9 (T11) (○). Each *point* represents the mean of number of trophozoites (calculated from triplicates). Deviations from the mean were generally *below* 3.50

Highest cytopathic effects were observed in strains 2HH (T4), 72/2 (T5), and ZOO9 (T11), all three completely lysing a monolayer of HEp-2 cells within 48 h. 2HH and 72/2 are old isolates that had been cultured axenically over long periods of time, while ZOO9 is a fresh isolate. All three of them are clinical isolates. 2HH was isolated from a keratitis patient, ZOO9 from tissue of a diseased snake, and 72/2 from the brain of a mouse. No cytopathic effects at all were shown by strains 4CL (T4), Pb30/40 (T7), and 4RE (T11), all old and nonclinical isolates. In general, cytopathic effects did not directly correlate to either growth rates in axenic or on plate culture. However, all strains without cytopathic effect were among the nonclinical isolates, and moreover, cytopathic effects were generally higher among clinical isolates.

Temperature tolerance was highest in strains DU08 (T4), 72/2 (T5), and ZOO9 (T11) (Table 3). Of these, 72/2 is an old isolate, while the other two are fresh isolates. All three of them are clinical isolates. Temperature tolerances did not strictly correlate to overall growth rates on plate cultures, although, all strains growing at 40°C also scored highest (+++) at 37°C and at 34°C. However, two strains, strain 2HH and strain SPA08, that showed equally high growth rates at 37°C did not grow at 40°C. Temperature tolerances did not also correlate to growth rates in axenic culture, e.g., strains NEFF and 9GU grew fast on plates at 34°C (Table 3) but were among the slowly growing strains with high generation times in axenic culture at RT (Fig. 1). In general, growth rates on agar plates did not correlate to growth rates in axenic culture.

As with cytopathic potential, all clinical strains showed the ability to migrate under agarose at least at 34°C, while of the nonclinical strains, two strains, Pb30/40 (T7) and

Table 3 Physiologic characteristics of investigated *Acanthamoeba* strains, listed according to origin and genotype

Genotype	Strain	Origin	Cytophatic effect	Growth rate on plate culture			Under-agarose migration	
			34°C	34°C	37°C	40°C	34°C	37°C
Clinical isolates								
T4	2HH	Keratitis patient, cornea	+++	+++	+++	−	+++	−
T4	1BU	Keratitis patient, cornea	++	+++	++	−	++	−
T4	3ST	Keratitis patient, cornea	+	++	+	−	++	−
T4	PAT06	Keratitis patient, cornea	+	+++	++	−	+	−
T4	SPA08	Keratitis patient, cornea	+	+++	+++	−	+++	+++
T4	DU08	Keratitis patient, cornea	+	+++	+++	+	+++	+++
T5	72/2	Mouse brain	+++	+++	+++	+++	++	++
T6	11DS	Keratitis patient, cornea	++	++	+	−	++	−
T11	ZOO9	Anaconda tissue	+++	+++	+++	+	++	+++
Nonclinical isolates								
T4	NEFF	Soil	+	+++	++	−	+++	−
T4	3250	Water	+	++	+	−	+++	+++
T4	4CL	Contact lens case	−	++	++	−	+	−
T4	9GU	Contact lens case	++	+++	−	−	+	−
T7	Pb30/40	Greenhouse	−	+++	+	−	−	−
T11	4RE	Contact lens case	−	++	++	−	−	−

4RE (T11), were not able to migrate under agarose neither at 34°C nor at 37°C; although on plate culture, they both showed high growth rates at these temperatures. Interestingly, all genotype T4 strains, clinical as well as nonclinical ones, showed under-agarose migration. Generally, the ability to migrate under agarose decreased with increasing temperature, and this was particularly obvious in old isolates. While the recent clinical isolates SPA08 (T4) and DU08 (T4) and also the recent nonclinical isolate 3250 (T4) showed similar under-agarose migration at 34°C and at 37°C, none of the old isolates, clinical as well as nonclinical, except for 72/2, showed under-agarose migration at 37°C—and this strain showed less under-agarose migration than all fresh isolates. Altogether, the ability to migrate in an under-agarose system correlated with the cytopathic effect. However, there was no correlation between the degree of under-agarose migration and cytopathic effect.

Discussion

Several *Acanthamoeba* strains of various genotypes can cause human infections, but different isolates within one genotype can possess different pathogenic potentials (Walochnik et al. 2000a, b; Xuan et al. 2008). Certain physiologic characteristics may indicate the pathogenic potential of a particular isolate, however, as shown in the current study, most of these are no permanent traits but alter over long-term culture; particularly, when cultured axenically at stable laboratory conditions. Traits that are not needed are lost or at least down-regulated as has recently also been shown by Koehsler et al. (2008, 2009) concerning encystment and protease activities.

This loss of certain traits was particularly obvious for temperature tolerance. Temperature tolerance is widely accepted as a prerequisite for pathogenicity in *Acanthamoeba* (Griffin 1972; Gianinazzi et al. 2009). The human eye has approximately 34°C and the human brain 37°C and strains that cannot grow at these temperatures most probably cannot cause disease. But this premise is not valid the other way around, meaning that not every strain that grows at 34°C/37°C can cause an infection of the eye/brain. Generally, T4 strains, both clinical and nonclinical isolates, prefer to grow at temperatures lower than 37°C (Booton et al. 2004) and indeed, in the current study, highest growth rates were observed at room temperature and 34°C, respectively. All strains, irrespective of origin or genotype, grew very well on *E. coli* at 34°C, and similar findings have been made by others (De Jonckheere 1991; Khan et al. 2002). This may be so because all investigated clinical isolates, except for 72/2, which actually was thermophilic, were either keratitis causing strains or from cold-blooded animals. For keratitis causing strains, the ability to grow at higher temperatures than 34°C is not relevant (Morton et al. 1991a, b) and indeed, strains isolated from keratitis cases but not growing any higher than 34°C are known (Booton et al. 2004). In axenic culture at room temperature used in most laboratories for long-term up keeping of strain collections, temperature tolerance is not

needed any longer and is lost or at least downregulated. *Acanthamoebae* are known to rapidly change their membrane lipid composition and induce delta 12-desaturase activities in response to abrupt temperature shifts (Jones et al. 1992, 1993). Long-term adaptions might be achieved by epigenetic regulation (Koehsler et al. 2008, 2009). However, multiple animal passages or human HEp-2 cell monolayer passages can restore altered physiological characteristics in *Acanthamoeba* (Mazur and Hadas 1994; Koehsler et al. 2009).

Growth rate per se also is a physiological characteristic corresponding to pathogenic potential (Walochnik et al. 2000b). It is generally plausible that more amoebae will cause more damage in the human body. An infection with a very slow growing (multiplying) strain will not lead to similarly high amoeba densities in the human body as an infection with a fast growing strain. Strains freshly isolated from clinical specimens usually divide very rapidly (Walochnik et al. 2000b; da Rocha-Azevedo and Costa e Silva-Filho 2007); however, this trait firstly need not be a permanent trait, and secondly, and this was also seen in the current study, strains that grow and divide rapidly on human cells need not necessarily also grow fast in either axenic culture or on agar plates in the presence of live bacteria. In this study for instance, the isolate with the lowest axenic growth rate, strain 2HH, showed a high cytopathic effect. However, as detected recently, this strain harbors an endosymbiont, and growth rate in this strain partly depends on the number of endocytobionts per cell (unpublished data). It is well known that endocytobionts of amoebae can significantly alter the growth rates of their hosts (Collingro et al. 2004).

Clinical isolates usually show cytopathic effects in cell cultures, whereas, nonclinical isolates are often unable to lyse human cell monolayers, and this also correlates to mouse pathogenicity (Cursons and Brown 1978; Walochnik et al. 2000b; da Rocha-Azevedo and Costa e Silva-Filho 2007). This is corroborated by our findings that all clinical isolates, irrespective of the genotype, exhibited cytopathic effects. All strains without any cytopathic effect were among the nonclinical isolates; however, three nonclinical isolates did have cytopathic effects. Although, of course, clinical isolates are usually pathogenic, nonclinical strains do not necessarily have to be nonpathogenic, as generally humans become infected rather by contact to *Acanthamoeba* trophozoites or cysts from the environment than by person to person contact (Ozkoc et al. 2008; Niyyati et al. 2009).

All clinical isolates, which were mostly keratitis isolates, were able to migrate under agarose at least at 34°C, the temperature of the human eye. Under-agarose migration was also observed in nonclinical isolates interestingly, particularly, in those strains exhibiting also cytopathic effects. The combination of both physiological characteristics may thus indicate a pathogenic potential as also observed previously (Thong and Ferrante 1986; Walochnik et al. 2000b).

Altogether, the results of our study indicate that there is no general difference in physiological characteristics between genotypes and origins of different *Acanthamoeba* strains. However, after long-term axenic culture, *Acanthamoeba* strains have adapted to laboratory conditions showing higher growth rates at the temperature used during axenic culture but losing their ability to grow or migrate under agarose at higher temperatures. To our opinion, this is very important when working with laboratory cultures.

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