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SHORT COMMUNICATION

Acanthamoeba strains lose their abilities to encyst synchronously upon prolonged axenic culture

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Abstract To evaluate the influence of prolonged axenic culture on the encystment capacity of *Acanthamoeba* spp., the encystment potential of four closely related *Acanthamoeba* strains, subcultured axenically for different periods of time, was evaluated comparing five encystment media. Media with more alkaline pH values were slightly more effective; however, the composition of the respective encystment medium had only limited influence on the encystment potential, while a strong correlation of losses in encystment potential and times strains had been cultured axenically was demonstrated. Furthermore, our results indicate that losses in encystment potential occur shortly after transfer into axenic culture to remain constant over many years.

Introduction

Potentially pathogenic free-living amoebae of the genus *Acanthamoeba* evade unfavourable environmental conditions by transforming into their highly resistant cyst stage. Cysts do not represent the infective stage but massive encystment is a characteristic stage of infections, and cysts are believed to be the source of recrudescences (Khunkitti et al. 1996).

In the laboratory, encystment of *Acanthamoeba* can be induced by replacing the nutrient medium with an encystment medium (Neff et al. 1964; Hughes et al. 2003). However, for studies on the nature of cysts, it is essential to produce synchronous cultures. Synchronous encystment is defined as >70% of cysts after 24 h (Neff et al. 1964).

In a preliminary experiment, it was observed that the reference strain *Acanthamoeba castellanii* Neff did not reach synchronous encystment in any of five different encystment media (own unpublished observation). Strain Neff is a very old strain that was isolated in 1957, and it has been regularly subcultured since then. In *Acanthamoeba* spp., adaptations to prolonged axenic culture, such as an attenuation of virulence (Mazur and Hadas 1994), changes in cellular enzyme activity and alterations in drug sensitivity, have been reported (Stevens and O'Dell 1974; Kasprzak et al. 1986). Therefore, it seemed conceivable that long-term culture might also affect the encystment potential. To prove this hypothesis, the encystment behaviour of four closely related *Acanthamoeba* strains, cultured axenically for different periods of time, was evaluated comparing also the different encystment media.

Materials and methods

Acanthamoeba castellanii strain Neff (ATCC No. 30010) and three strains isolated from cornea specimens of keratitis patients in Austria, namely strain 1BU, strain PAT05 and strain PAT06, isolated in 1998, 2005 and 2006, respectively, were used in this study. All these strains had been subcultured in proteose–peptone–yeast (PYG) medium regularly since their initial isolation.

These strains were chosen because of their close genetic relationship all belonging to genotype T4. Strain 1BU had been genotyped earlier (Walochnik et al. 2000); for the other three strains, strains Neff, PAT05 and PAT06, 18S rDNA sequencing was performed as described previously (Walochnik et al.

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Table 1 Percentages of encysted cells of strains NEFF, 1BU, PAT05 and PAT06 in tested encystment media and with PYG as control

Encystment medium	Strain	% of encysted cells (± SEM) after			
		1 h	24 h	48 h	72 h
AMPL	NEFF	2.7±2.2	38.0±6.8	59.9±4.0	68.4±4.4
	1BU	2.6±0.9	38.8±6.0	52.1±3.4	60.3±2.6
	PAT05	0.7±1.2	61.9±2.9	70.2±5.2	81.9±3.3
	PAT06	10.0±1.6	97.8±1.9	98.9±1.3	99.5±0.8
Tris	NEFF	2.0±0.9	41.6±9.3	59.4±6.6	71.6±6.9
	1BU	2.9±0.9	50.9±6.1	65.8±7.1	74.4±4.0
	PAT05	1.1±0.9	83.8±4.0	89.1±5.0	94.5±2.8
	PAT06	9.6±1.3	97.1±2.7	98.5±1.7	99.6±1.1
Taurine	NEFF	3.0±2.0	40.2±6.9	64.4±5.5	69.0±4.5
	1BU	2.2±1.3	43.9±6.1	53.6±2.3	61.1±2.7
	PAT05	0.9±0.8	52.9±6.8	62.8±5.5	73.3±2.7
	PAT06	11.3±1.6	96.7±3.2	97.6±1.1	98.8±1.3
McMillen	NEFF	2.6±1.5	39.1±6.4	49.3±8.8	58.0±9.1
	1BU	2.1±1.1	35.8±5.4	59.0±4.0	67.5±5.2
	PAT05	1.4±1.0	44.2±2.9	55.1±4.7	68.7±3.2
	PAT06	10.0±2.0	93.9±0.9	94.9±1.7	96.0±2.7
$MgCl_2$	NEFF	2.5±1.8	23.7±4.7	40.4±4.1	47.4±7.7
	1BU	2.7±1.6	34.6±6.5	59.2±5.0	65.7±4.2
	PAT05	0.6±0.7	41.1±6.5	64.2±7.4	71.8±5.6
	PAT06	9.5±1.4	97.2±3.3	96.9±2.4	98.4±1.4
PYG	NEFF	2.5±0.9	1.9±1.4	3.9±2.4	4.9±2.5
	1BU	2.2±1.1	3.1±0.8	4.2±1.2	6.4±1.9
	PAT05	1.0±0.7	1.9±1.2	4.0±1.4	7.8±2.0
	PAT06	10.1±1.9	12.0±7.6	18.0±4.5	23.1±4.6

2000). Sequence data were deposited in GenBank and are available under the following accession numbers: EF429130 (PAT05), EF429131 (PAT06) and EF554328 (Neff).

The encystment media used in this study are referred to as AMPL medium (pH 8.8; Neff et al. 1964), tris medium (pH 9.0; Hirukawa et al. 1998), taurine medium (pH 8.0; Hughes et al. 2003), $MgCl_2$ medium (pH 7.0; Griffith 1970) and McMillen medium (pH 6.8; McMillen et al. 1974). Encystment was induced as described previously (Hughes et al. 2003). PYG medium served as control. Progress of encystment was evaluated by counting cells at the beginning and 24, 48 and 72 h after induction of encystment using a Fuchs–Rosenthal hemacytometer. Three independent experiments were carried out, each with triplicate samples.

Viability of cysts was evaluated after keeping the cysts in the respective encystment medium for 15 days. To kill immature cysts and remaining trophozoites, cysts were treated with 3% HCl overnight. After transfer into PYG medium, re-emergence of trophozoites was monitored.

Results and discussion

It was shown that the activation of the encystment machinery of the investigated *Acanthamoeba* strains is very much dependent on two things: firstly, on the time a strain has been cultured axenically, and secondly, on the choice of the respective encystment medium. Cyst counts for all strains in all media are shown in Table 1.

Sequencing revealed that all strains represent genotype T4 and thus are closely related. However, significant differences in the encystment capacity of these strains were

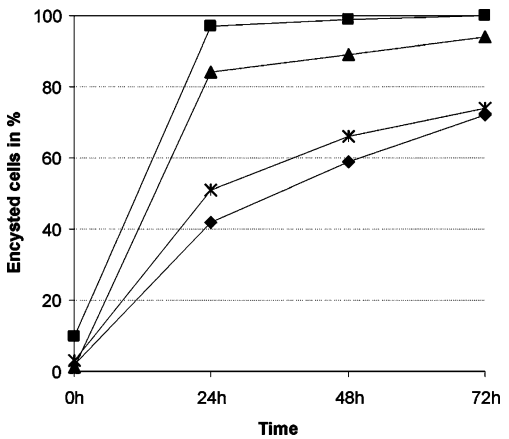

Fig. 1 Percentages of encysted cells for strain Neff (*diamonds*), strain 1BU (*asterisks*), strain PAT05 (*triangles*) and strain PAT06 (*squares*) in tris medium

observed, strongly correlating to the time they had been cultured axenically. The "older" strains Neff and 1BU did not encyst synchronously in any of the encystment media tested. After 24 h, cyst rates were approximately 40% for both strains. Even after 72 h, cyst rates lay below 75%. Strain PAT05, cultured axenically for approximately 6 months, only encysted synchronously in tris medium, but it generally exhibited slightly higher encystment rates. Strain PAT06, transferred to axenic medium only a few weeks prior to encystment experiments, did not only show cyst rates above 96% already after 24 h in all media tested but also elevated cyst rates throughout culture and in the control medium.

These results show that a fresh isolate like strain PAT06 readily encysts after nutrient depletion, while a slightly older strain like PAT05 exhibits a significantly lower encystment potential. No synchronous encystment was observed for the extensively subcultured strains Neff and 1BU, which showed similar encystment potentials, despite their different times in axenic culture (i.e. 41 years!).

Tris medium appeared to be the most effective medium for all four strains (Fig. 1). While, for strain Neff and 1BU, tris medium was just slightly more effective than other media, it was the only medium in which PAT05 exhibited synchronous encystment. For strain PAT06, all media were comparable with more than 96% of cysts within 24 h. Interestingly, the effects of the five media differed in all strains, suggesting that there are subtle differences in the way each strain is responding. In all five encystment media, cysts reached maturity and stayed viable for at least 15 days. After treatment with 3% HCl, all strains emerged from the cysts within 24 h.

In summary, the observed encystment rates of the investigated strains indicate that the decline in synchronous encystment potential arises after only a few months in axenic culture. Our findings indicate that, for several years, the encystment potential decreases further upon extensive subculturing to remain constant after a certain period of time. For older strains with impaired encystment potential, apparently media with highly alkaline pH values are more effective, while a fresh isolate obviously responds equally to all media.

Our hypothesis is that, under axenic conditions, *Acanthamoeba* trophozoites readily adapt to the perfect environment they are confronted with. Constant temperature and pH in combination with a nutrition surplus may lead to a down-regulation of genes that are no longer required. Because encystment is a survival strategy of acanthamoebae and is induced under inhospitable environmental conditions in natural habitats, it might be negligible in a habitat where survival is more or less guaranteed. Shutting off mechanisms that become unessential under changed environmental conditions has been reported for several single-cell organisms. Examples are the irreversible loss of virulence and encystment potential in *Entamoeba histolytica*, marked reductions in locomotive ability of *Naegleria fowleri* or changes in the expression patterns of some proteinases in *Trichomonas vaginalis* isolates after prolonged laboratory culture (Phillips 1973; Thong and Ferrante 1986; Neale and Alderete 1990). Gene silencing as a result of environmental regulation of gene expression does strongly indicate the involvement of heritable, yet reversible, epigenetic mechanisms. This theory is supported by the fact that the loss of virulence in *Acanthamoeba* is reversible by passage through animal tissues (Mazur and Hadas 1994).

Altogether, it was demonstrated that *Acanthamoeba* encystment is initiated most effectively by media with highly alkaline pH values and that a strain's encystment capacity is very much dependent on the time the strain had been cultured axenically. The exact mechanisms involved in these processes remain to be defined. The fact that prolonged axenic culture leads to losses in several pathogenicity associated characteristics in *Acanthamoeba* might certainly influence the outcome of studies on the pathogenic potential and drug susceptibility of *Acanthamoeba* strains.

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