Excystment of Axenically Prepared Cysts of *Hartmannella culbertsoni*

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SUMMARY

Axenically prepared cysts of *Hartmannella culbertsoni* readily excysted in the presence of heat stable factors prepared from *Escherichia coli*, *Klebsiella aerogenes*, *Staphylococcus aureus*, *Sarcina lutea*, *Bacillus subtilis*, *Bacillus megaterium* and several fungi. Peptone, proteose peptone, tryptone or amino acids also promoted excystment. Crowding of the cysts and dilution of bacterial extracts adversely affected the excystment. Continual presence of the factors in the medium was essential for excystment.

INTRODUCTION

Many different environmental factors can trigger the excystment of amoebae (Singh, 1973). The presence of bacteria appears to be essential for the process (Beers, 1946; Crump, 1950; Hall, 1953) and the bacterial strains that permit excystment show a certain degree of selectivity (Dudziak, 1955; Singh, Das & Saxena, 1963). Following the suggestion of Crump (1950) that the above triggering action may be associated with some metabolic products of bacteria, Singh, Mathew & Anand (1958) and Singh, Mathew & Sreenivasaya (1956) prepared a heat-labile, dialysable factor from a *Klebsiella (Aerobacter)* sp. and a heat-stable, water-soluble factor from the same organism and also *Escherichia coli*. Part of the excystment-promoting activity was due to amino acids present in these extracts, and indeed exogenously added amino acids did promote the excystment of amoebic cysts (Drozanski, 1961; Jeffries, 1962; Singh, Datta & Dutta, 1971; Singh, Saxena & Iyer, 1965).

Most previous studies used cysts prepared in the presence of either monobacterial or mixed cultures after the cysts had been exposed to HCl, trypsin or detergents to remove bacteria (Singh *et al.*, 1965, 1971). Cysts exposed to HCl, detergents or enzymes probably retain their viability but change their permeability (Imam, Dutta & Agarwala, 1968). The question as to how far these changes affect subsequent excystment has not been unequivocally answered.

Hartmannella culbertsoni can grow in a medium without bacteria and the resulting trophozoites can be transformed into mature viable cysts in a liquid medium containing NaCl, MgCl₂ and taurine (Kaushal & Shukla, 1975). Such cysts do not require any pre-treatment since they are not contaminated with live or dead bacteria and hence provide an ideal starting material for studying the biochemistry of excystment. This paper describes some studies on the excystation of cysts formed from axenically grown *H. culbertsoni* induced by cell-free extracts of a few bacteria, fungi, growth media and amino acids.

METHODS

Growth and harvesting of cells. *Hartmannella culbertsoni* (Culbertson strain A-1; Singh & Das, 1970) was obtained from Dr. B. N. Singh of this Institute and grown axenically in medium containing: peptone (2 %, w/v); NaCl (0.5 %, w/v); thiamin, 10 mg l⁻¹; and cyanocobalamin, 5 µg l⁻¹ – as described earlier (Kaushal & Shukla, 1975).

Encystment. Encystment was induced in a liquid medium containing 15 mM-MgCl₂, 20 mM-aurine and 86 mM-NaCl. The above medium (300 ml) was dispensed into 1 l Erlenmyer flasks, sterilized and inoculated with freshly harvested amoebae (10 ml; 3×10^8 cells) and incubated with shaking at 28 ± 2 °C. Transformation of trophozoites into cysts was observed microscopically as described previously (Kaushal & Shukla, 1975). After 4 to 5 days, the cysts were collected, kept at 4 °C for 2 days and then washed several times by dispersing in sterile 150 mM-NaCl, centrifuging at 500 g for 3 min and finally suspending in 150 mM-NaCl. When stored at 4 °C, no adverse effect was noticed on excystment for up to one month of storage.

Preparation of bacterial and fungal extracts. *Escherichia coli*, *Klebsiella (Aerobacter) aerogenes*, *Staphylococcus aureus*, *Sarcina lutea*, *Bacillus subtilis* and *Bacillus megaterium* were grown in nutrient broth, harvested by centrifuging and an extract prepared by the method of Singh, Mathew & Anand (1958). The aqueous extract of bacteria containing the heat-stable, water-soluble components was freeze dried, and reconstituted in 0.5 % NaCl to yield a 1 % solution (10 mg ml⁻¹) of excystment factor(s). *Aspergillus niger*, *Chaetomium globosum*, *Myrothecium verrucaria*, *Trichoderma viride* and an unidentified isolate F₁ were grown in a medium at pH 5.0 containing (g l⁻¹): glucose, 10; peptone, 5; KH₂PO₄, 1; and MgSO₄·7H₂O, 0.5. Fungal extracts were prepared by the method of Jehan *et al.* (1975).

Excystment procedure. Excystment was carried out in tubes (25 × 150 mm) containing 4.5 ml of sterile NaCl (0.5 %), the indicated amount of the agents under test and 0.5 ml cyst suspension (5×10^5 cysts ml⁻¹). The tubes were shaken at 35 ± 2 °C. The hatching of double walled cysts of *Hartmannella culbertsoni* took place in two stages. First the amoebae shrank and moved within their cyst wall (intermediate form). Excystment was complete when the trophozoite came through an opening in this wall. At this stage the cyst wall or the empty cyst was still visible. Samples were taken aseptically at intervals and counts were made of all forms using a haemocytometer. Samples were taken at the end of the experiment and inoculated into nutrient broth to check for possible bacterial growth though in no case was there any contamination.

RESULTS

Excystment of H. culbertsoni in growth medium and in the presence of an E. coli extract

Excystment of *H. culbertsoni* occurred in the presence of an extract of *E. coli* to the same extent as it did in a liquid growth medium (Table 1). During excystment the number of amoebae (cysts plus intermediate forms plus trophozoites) did not increase either in the *E. coli* extract or in the growth medium. *E. coli* extract does not support the growth of amoebae (Singh *et al.*, 1958) up to 72 h. In medium containing *E. coli* extract, the number of cysts emerging at 48 h was more but the total number of amoebae remained the same. The number of empty cysts, however, was obviously not identical with the number of trophozoites due to degradation of empty cysts presumably by enzymes secreted by the amoebae (Kaushal & Shukla, 1976). In growth medium, however, the number of trophozoites increased as a result of not only more cysts hatching but also due to multiplication of the

Table 1. *Excystment in growth medium and E. coli extract*

Time (h)	$10^{-3} \times \text{No. ml}^{-1}$ in growth medium*				$10^{-3} \times \text{No. ml}^{-1}$ in <i>E. coli</i> extract (10 mg ml ⁻¹)†			
	Cysts	Intermediate forms	Trophozoites	Empty cysts	Cysts	Intermediate forms	Trophozoites	Empty cysts
0	500	—	—	—	500	—	—	—
24	110 ± 27	60 ± 13	330 ± 39	330 ± 35	60 ± 16	110 ± 23	330 ± 40	330 ± 52
48	80 ± 20	30 ± 10	450 ± 34	290 ± 34	40 ± 10	40 ± 8	420 ± 47	350 ± 33

Initial viability of cysts, 97.5 %.

* Growth medium contained peptone (2 %), cyanocobalamin (5 µg l⁻¹) and thiamin (10 mg l⁻¹).

† *E. coli* extract was prepared as described in Methods.

Table 2. *Excystment with different concentrations of cysts*

The excystment medium was *E. coli* extract (10 mg ml⁻¹). Data given are the means for triplicate samples taken from two separate experiments.

Initial no. of cysts ml ⁻¹	Percentage excystment after					
	4 h	8 h	12 h	16 h	20 h	24 h
1×10^5	6	17	29	42	63	81
5×10^5	4	11	21	30	52	67
1×10^6	3	7	18	25	43	51
5×10^6	3	7	15	23	37	47

trophozoites. Thus, there was a 12 % increase in the total number of amoebae at 48 h but the number of empty cysts decreased due to degradation. The count of trophozoites and empty cysts at 24 h therefore gives a good indication of the degree of excystment. Since most of the excystment described here was done using only bacterial extract the counts of trophozoites at 48 h represent an accurate estimation of the degree of excystment. In growth medium at 48 h, the multiplication of amoebae was likely to have vitiated the result of the count. However, the haemocytometer counts up to 24 h during which there was no multiplication of trophozoites, would represent the extent of excystment.

Factors affecting the excystment of H. culbertsoni

E. coli extract. This readily promotes the excystment of axenically prepared cysts of *H. culbertsoni*. The extract caused 50 % excystment at a 1:1600 dilution and still gave 35 to 40 % excystment at 1:12800 dilution.

Cyst concentration. Excystment depended upon the concentration of cysts in the excystment medium (Table 2). There was extensive excystment within 24 h with 1×10^5 cysts ml⁻¹ but with 5×10^6 cysts ml⁻¹ only 47 % of cysts had excysted. The rate of excystment was linear until it reached the maximum at about 24 h.

Time of exposure of the cysts to E. coli extract. Cysts were incubated in *E. coli* extract for different periods and were then washed free of the extract. They were then incubated in 0.5 % sterile NaCl and the degree of excystment measured at 24 and 48 h intervals. The results (Table 3) suggest that the factors have to be present throughout for bringing about excystment.

HCl, sodium lauryl sulphate and trypsin. The effect of treatment with different concentrations of sodium lauryl sulphate and HCl on the excystment and viability of cysts as judged by eosin staining was studied. Low concentrations (0.1 to 1 %) of HCl and sodium lauryl sulphate did not affect the cysts up to 24 h exposure. However, treatment with 2 % HCl or

Table 3. *Effect of E. coli extract on excystment of H. culbertsoni*

The cysts were pre-incubated for various periods in *E. coli* extract (10 mg ml^{-1}) and then transferred to 0.5 % NaCl and incubated for 48 h. Data are the means of counts in a haemocytometer for triplicate samples taken from two separate experiments.

Pre-incubation time of cysts in the <i>E. coli</i> extract (h)	Relative % of forms of organism after					
	24 h			48 h		
	Cysts	Intermediate form	Trophozoite	Cysts	Intermediate form	Trophozoite
0	100	0	0	100	0	0
4	49	45	5	49	45	6
8	47	42	11	47	42	11
12	37	42	21	37	42	21
24	20	11	69	20	11	69
48	10	14	76	10	9	81

2 % lauryl sulphate for 24 h adversely affected the viability of cysts. Treatment of cysts as such with trypsin (1 %, 100 mM-Tris buffer, pH 7.6) for 10 min enhanced excystment. When HCl-treated cysts were subjected to trypsin treatment, excystment was increased from 67 to 83 %.

Different bacterial and fungal extracts. Aqueous extracts of *B. subtilis*, *B. megaterium*, *Sarcina lutea* and *S. aureus* promoted 65 to 80 % excystment after 24 h exposure and 85 to 90 % after 48 h exposure. Extracts from *A. niger*, *C. globosum*, *T. viride* and *M. verrucaria* also promoted good excystment.

Growth medium and protein hydrolysate. The growth medium for *H. culbertsoni* gave good excystment. Peptone (1 to 2 %) itself was also effective but adding thiamin and cyanocobalamin had no significant effect (Table 4). Other proteinaceous substrates, such as tryptone or proteose peptone, were equally effective in promoting excystment.

Amino acids. Different amino acids (10^{-2} to 10^{-3} M) caused varying degrees of excystment of axenically prepared cysts of *H. culbertsoni* (Table 5). Glutamic acid was the most potent followed by serine, threonine and alanine. Excystment was low with the basic and aromatic amino acids. No linear or quantitative relationship was observed between the concentrations of amino acids and the degree of excystment.

DISCUSSION

Factors causing excystment seem to be of wide and almost ubiquitous occurrence. Peptones and protein hydrolysates, and extracts from several Gram-positive and Gram-negative, sporulating and non-sporulating bacteria promoted this activity. Fungal extracts also promoted excystment. Since the growth of amoebae as well as encystment took place under sterile conditions and sterility was also maintained during excystment, the excystment activity of the media employed cannot be ascribed to bacterial contaminants. The excystation mediated by cell-free extracts of bacteria and fungi must be associated with some chemical entities present in them. That amino acids are among the important agents that cause excystment is to be inferred from their presence in cell-free extracts as well as in peptones. The earlier studies of Singh *et al.* (1958) and rigorous fractionation of *E. coli* extract (Kaushal, Shukla & Krishna Murti, unpublished observation) suggest that the excystment promoting activity of cell-free bacterial extracts may indeed be due to the presence of amino acids.

Table 4. Excystment of *H. culbertsoni* in different media

Data are the means of counts in a haemocytometer for triplicate samples taken from two separate experiments.
The initial concentration of cysts was $5 \times 10^5 \text{ ml}^{-1}$.

Medium	Relative % of forms of organism after					
	24 h			48 h		
	Cysts	Intermediate forms	Trophozoite	Cysts	Intermediate forms	Trophozoite
Sodium chloride (0.5 %)	100	0	0	100	0	0
<i>E. coli</i> extract (10 mg ml^{-1})	12	21	67	9	7	84
Peptone (2 %) + thiamin ($1 \text{ mg } 100 \text{ ml}^{-1}$) + cyanocobalamin ($0.5 \mu\text{g } 100 \text{ ml}^{-1}$)	27	11	62	15	7	78
Peptone 2 %	22	19	59	17	4	79
1 %	24	18	58	16	7	77
0.5 %	52	23	25	52	10	38
0.1 %	61	30	9	50	39	11
Peptone (2 %) + thiamin ($10 \text{ mg } 100 \text{ ml}^{-1}$)	21	18	61	18	4	78
Peptone (2 %) + cyanocobalamin ($0.5 \mu\text{g } 100 \text{ ml}^{-1}$)	19	24	57	13	6	81
Tryptone (2 %)	18	21	61	19	9	72
Protease peptone (2 %)	16	19	65	14	5	81
Protease peptone (1 %) + tryptone (1 %)	17	16	67	12	6	82

Table 5. *Effect of amino acids on excystment of H. culbertsoni*

Cysts at 5×10^5 ml⁻¹ were incubated for 24 h. *E. coli* extract (10 mg ml⁻¹) as the standard gave 67 % excystment; NaCl (0.5 %) as the control gave none. The pH of the amino acid solutions was adjusted to 6.5–7.0; similar results were obtained using the amino acids at 10^{-2} M. Data are the means of counts in a haemocytometer for triplicate samples taken from two separate experiments.

Amino acids (10^{-3} M)	Excystment (%)	Amino acids (10^{-3} M)	Excystment (%)
β -Alanine	30	L-Lysine	21
DL-Alanine	29	DL-Methionine	12
L-Arginine.HCl	2	DL-Methionine sulphoxide	21
L-Asparagine	21	DL-Norleucine	7
DL-Aspartic acid	28	DL-Norvaline	27
L-Aspartic acid	20	DL-Ornithine.HCl	13
L-Cysteic acid	19	L-Ornithine.HCl	19
L-Cysteine.HCl	15	L-Phenylalanine	21
L-Cystine	31	L-Proline	8
DL-Glutamic acid	43	DL-Serine	32
L-Glutamic acid	39	L-Serine	31
L-Glutamine	28	DL-Threonine	32
Glycine	23	L-Threonine	34
L-Histidine.HCl	13	DL-Tryptophan	13
Hydroxy L-Proline	23	L-Tryptophan	11
Isoleucine	14	DL-Tyrosine	14
DL-Leucine	18	L-Tyrosine	11
L-Leucine	8	DL-Valine	31
DL-Lysine.HCl	19		

Treatment of cysts with HCl and trypsin accelerates excystment. This may be either due to increased permeability of cysts or to the unmasking of receptor sites for the excystment agents.

Excystment in the cysts of *H. culbertsoni* was noticed even with very low concentration of the factor although higher concentrations were required for quantitative excystment. No linear relationship was observed between the amount of excystation agent and the degree of excystment. Whether the induced excystment was preceded by uptake and binding of the factor responsible could not be discerned from the present studies. A high concentration of cysts leading to crowding of the environment adversely affected excystment.

Continuous presence of excystment factor was essential and removal of the agent stopped further excystment. This may indicate the absence of a trigger mechanism in excystment. Alternatively, on the basis of the asynchronous nature of the cyst population, it may be that all cells do not respond to the triggering action of the agents simultaneously.

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