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Mutant with heat-sensitive capacity for phagocytosis in tetrahymena: isolation and genetic characterization

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SUMMARY

A mutant of *Tetrahymena* with heat-sensitive phagocytosis was obtained using a tantalum-particle enrichment procedure. The mutant phenotype is most likely determined by a somatic (macronuclear) mutation(s). The inability of the mutant to sustain cell division and to phagocytize at 37 °C are most likely determined by the same mutation. The phenotype of the mutant is stably inherited under vegetative propagation at 30 °C. At 37 °C, the mutation affects the development of the oral apparatus, the phagocytotic organelle. This mutant has proven useful for the study of cellular functions related to phagocytosis.

1. INTRODUCTION

Ciliates are organisms well suited for the study of phagocytosis (i.e the uptake of particulate matter into cytoplasmic vacuoles), as this process occurs at a unique, specialized site, the oral apparatus (OA). It is a structure composed of cilia, basal bodies, microtubules and other fibres, organized in a manner designed to collect particles in the cytopharynx, and to form phagosomes (food vacuoles) sequentially and actively. During vegetative growth, a new OA must be developed for the posterior daughter at every cell division. Thus, the OA is an interesting structure from the standpoint of both the physiology of phagocytosis and cellular morphogenesis.

The study of the development and physiology of the OA in *Tetrahymena* may be approached by means of genetic dissection, i.e. the isolation and characterization of mutants having defects in these processes. *Tetrahymena* is an organism well suited for this study because its OA is partially characterized at the EM level (Williams & Zeuthen, 1966; Nilsson & Williams, 1966) and at the biochemical level Gavin, 1974). In addition *Tetrahymena* is well suited for genetic dissection (Orias & Bruns, 1974).

Since phagocytosis is required for growth of *Tetrahymena* in proteose-peptone medium (Rasmussen, 1973) we decided to search for mutants with heat-sensitive

defects in phagocytosis. In this paper we report on the isolation and genetic characterization of a mutant with temperature-sensitive development of the OA. Its physiological characterization is reported in a separate publication (Orias & Pollock, 1975).

2. MATERIALS AND METHODS

(i) *Strains*

The strains used in this study are all derived from inbred strain D-1968, originally obtained from Dr D. L. Nanney (University of Illinois). Strain CHX-F3 is homozygous for a dominant mutant allele (*Chx*-1) determining resistance to cycloheximide (CHX) (Roberts & Orias, 1973*b*). Strain CHX-SS has a heterozygous germ line (micronucleus), *Chx*-1/*Chx*$^+$, but has a cylcoheximide-sensitive phenotype. This strain is a functional heterokaryon (Bruns & Brussard, 1975) and was generated by vegetative phenotypic assortment (see Sonneborn review, 1974) from a cycloheximide-resistant heterozygote. Standard mating-type tester strains were also obtained from Dr Nanney. The strains are permanently stored under liquid nitrogen (Simon & Flacks, 1974).

(ii) *Media*

Clones were maintained, grown and tested in our standard PPY + PS medium. This is a 2 % proteose peptone, 0·1 % yeast extract and salts medium, supplemented just before use with 250 μg/ml each of penicillin G and streptomycin sulphate (Orias & Flacks, 1973). For tests of cycloheximide resistance or selection of CHX-resistant progeny, the basic PPY + PS medium was supplemented just before use with 10 μg/ml of cycloheximide (CHX-10 medium). (One mg/ml aqueous stock solutions of CHX were made just before use, or were kept frozen and used not more than 3 days after preparation.) Food vacuole formation tests used PPY + PS medium containing 0·5 % (v/v) indian ink (Higgins). The composition of other specialized media used in crosses and mating-type tests was previously described (Orias & Flacks, 1973).

(iii) *Routine methods*

Methods for culturing, cell counting, replica subcloning, mutagenesis, crossing, testing for mating type and for CHX resistance have been described in detail (Orias & Flacks, 1973; Roberts & Orias, 1973*a*, *b*; Orias & Bruns, 1974).

(iv) *Phagocytotic competence*

A simple, reliable and efficient method was designed to test the ability of large numbers of progeny clones to form food vacuoles (phagocytize). The clones to be tested were replicated to an appropriate array of drops (of *c.* 30 μl) of PPY + PS medium on each of two Petri plates. One plate was incubated at 30 °C, the other at 37 °C. On the next day, approximately 5 μl of PPY + PS medium, containing 2 % indian ink, were added to each drop, with a pipette drawn out to a fine tip. The plates were incubated at the corresponding temperatures (30 or 37 °C). After 1 h of

incubation with ink, the Petri plates were carefully turned upside down and were scored under the 10× objective of a compound microscope. Cultures of both parental strains were included as controls in all tests.

(v) *Enrichment for a mutant with heat-sensitive phagocytosis*

The overall strategy consisted of (*a*) mutagenizing, to increase the presumably low spontaneous frequency of such mutants; (*b*) crossing the cells, to allow expression of the mutant genes in the new macronuclei; (*c*) vegetative propagation of the exconjugant mixture in mass culture for many generations, to encourage the build-up of recessive mutant alleles in the macronucleus (through random distribution, see Discussion); (*d*) physical enrichment for the mutants expressing the desired phenotype, and (*e*) individual isolation of potential mutants. The experimental details are described below.

(*a*) *Mutagenesis and conjugation*

A 50 ml culture of mating type V wild-type strain D was mutagenized with 10 μg/ml of *N*-methyl, *N'*-nitro, *N*-nitrosoguanidine (NG) for 6 h at 30 °C as previously described (Orias & Flacks, 1973). After washing the cells and resuspending in an equal volume of Dryl's salts medium, they were mixed with an equal number of similarly grown and washed cells of mating type III, strain D (except that NG treatment was omitted). The mixture, containing 5×10^5 cells/ml, were incubated overnight at room temperature and conjugated. On the next day the mixture of progeny and unconjugated parental cells was added to 500 ml of PPY + PS medium in a 4 l Erlenmeyer flask and incubated at 30 °C.

(*b*) *Vegetative propagation of the mutagenized progeny*

Vegetative propagation of the culture was performed by making daily serial transfers of 50 ml from the previous culture into 500 ml of PPY + PS medium in a 4 l Erlenmeyer flask. The culture was incubated at 30 °C.

(*c*) *Physical enrichment for the desired mutant in mass culture*

At various times during propagation of the mutagenized cultures, samples were taken to start 'mutant enrichment' subcultures. Each enrichment subculture was subjected to a series of enrichment cycles designed to increase the frequency of the desired mutant in the subculture, using a modification of the method of Wolfe (1973).

Each cycle of enrichment consisted of the following steps. A 5 ml sample from the previous enrichment cycle was added to 50 ml of PPY + PS in a 250 ml Erlenmeyer flask and incubated at 37 °C, to inactivate phagocytosis in the presumptive mutants. After overnight incubation, 5 ml aliquots of the culture were distributed to Petri plates and an equal volume of PPY + PS medium, containing 5 mg/ml of tantalum powder, was added to each plate; incubation was continued at 37 °C for 10 min. The cell and tantalum suspension was then spun for 2 min at

approximately 310 *g* in a clinical centrifuge. The pellet was re-suspended in 10 ml of PPY + PS medium and spun again for 2 min. The second pellet, re-suspended in 1 ml of PPY + PS medium, and two 0·5 ml rinses of the tube were carefully added to the top of a discontinuous Ficoll gradient, formed by the superposition of 2 ml of 20 %, 1·5 ml of 15 %, and 1·5 ml of 10 % Ficoll (Pharmacia), all v/w, contained in a conical 15 ml centrifuge tube. The gradient was then spun for 2 min at 310 *g*. The layers were carefully transferred to separate tubes with a Pasteur pipette, and samples were examined microscopically for the number and size of tantalum-containing vacuoles. The layer(s) containing less than 10 % vac formers was added to 50 ml PPY + PS medium. This layer typically contained about 5 % of the tantalum-treated cells. The culture was then incubated at 30 °C. After 2 days, the next cycle of enrichment was initiated.

The first cycle of enrichment differed from all the others only in that (*a*) the initial sample came from a serial transfer of the culture of mutagenized cells, (*b*) the cycle was initiated by taking a 50 ml sample, adding it to 500 ml of PPY medium at 37 °C and (*c*) a 100 ml sample of 37 °C was fed with tantalum.

(*d*) *Individual isolation of potential mutants*

After five serial cycles of enrichment as described above, a more direct search for the mutant was initiated. A 5 ml sample from the 5th cycle 30 °C culture was added to 50 ml of PPY + PS medium and incubated overnight at 37 °C. Next day, 1 ml samples of the cultures were mixed with 1 ml of PPY + PS medium containing 0·5 % indian ink in a small (35 × 10 mm) Petri plate. After ½–1 h of incubation at 37 °C the mixture was observed at room temperature under a dissecting microscope, and white (vac-less) cells were individually isolated with a micropipette to separate drops in a Petri plate. The drop cultures were incubated at 30 °C, and subsequently re-tested for their capacity to form vacuoles at 30 and 37 °C. Many single cell isolations were made from the clones that passed this test, and each was re-tested in the same manner. Strain NP1 was isolated by this method.

(vi) *Mass selection of progeny*

In one cross used in the genetic analysis of strain NP1, progeny were selected in mass culture. Strain NP1, which is homozygous for cycloheximide sensitivity, was crossed to strain CHX-SS, whose germ line is heterozygous for dominant CHX-resistance but which also has the sensitive phenotype, as described above. One half of the progeny of this cross are expected to be CHX-resistant. Parental cells and cells which conjugated but did not develop a new macronucleus ('non-conjugants'), which are still CHX-sensitive, were eliminated (along with the CHX-sensitive progeny class) by the following procedure (Roberts & Orias, 1973*b*; Bruns & Brussard, 1975). The conjugating mixture was diluted 10-fold with PPY + PS medium 6 h after being prepared and was incubated at 30 °C. On the next day a sample of the culture was diluted 10-fold with CHX-10 medium and incubated at 30 °C. Three days later, sensitive cells had died. Single cells were then isolated.

(vii) *Isolation of wild-type revertants of strain NP1*

A culture of the mutant strain NP1 was mutagenized with NG for 6 h, as previously described. The mutagen was washed, and a 10-fold dilution of the cells in PPY + PS medium was made. The culture was incubated at 30 °C, to allow an opportunity for expression of the wild-type phenotype. On the next day, a sample of the culture was diluted 10-fold and incubated at 37 °C, to select revertants. After 4 days at 37 °C the mutagenized culture had become turbid with growth. By contrast, cultures which were not mutagenized, but were otherwise identically treated, did not become turbid after prolonged incubation at 37 °C. Cells from each revertant culture were tested for vacuole forming ability. At 37 °C, cells from the turbid mutagenized culture were *vac*$^+$, while the survivors in the non-mutagenized culture were *vac*$^-$.

3. RESULTS

(i) *Isolation of a mutant with heat-sensitive phagocytosis*

Using the methods described in the previous section, we isolated a mutant strain which we have designated NP1. This strain has the following properties (Orias & Pollock, 1975). At 30 °C, growth and phagocytosis in strain NP1 are essentially normal. After overnight incubation at 37 °C, more than 95 % of the cells in the culture do not perform phagocytosis. We call this the *ts vac*$^-$ phenotype (for temperature-*s*ensitive food *vac*uole-less). Such cells are also incapable of further cell division in PPY + PS medium at 37 °C. The mutant cells at 37 °C show severe abnormalities of the OA. These phenotypes are reversible, as expected from the conditions used to enrich for the mutant; that is, if those cells are transferred back to 30 °C, more than 90 % of them recover the ability to phagocytize and divide. The next section described the results of our genetic analysis of the mutant strain.

(ii) *Genetic analysis*

Strain NP1 was crossed to strains CHX-SS and CHXF3 to study the inheritance of the *ts vac*$^-$ phenotype. Resistance to 10 μg/ml CHX was also examined as a check for normal nuclear segregation.

The results of these crosses are shown in Table 1. In crosses I and II conjugating pairs were isolated and the two exconjugants of each pair were separated. Only immature, viable pairs were scored. In cross III, progeny were selected in mass culture by eliminating non-conjugant cells of parental phenotype with post-conjugational cycloheximide treatment (see Materials and Methods section). CHX-resistant progeny were then singly isolated and tested. No F_1 progeny expressed the *ts vac*$^-$ phenotype in any of the above three crosses, either soon after conjugation or 50 fissions later. CHX-resistance showed the expected 1 : 1 segregation in crosses I and II. These results rule out simple cytoplasmic mutation or a dominant mutation present also in the germ line as the basis of the NP1 phenotype.

In order to determine if the *ts vac*$^-$ phenotype of strain NP1 could be determined by a recessive mutation, 36 CHX-resistant F_1 progeny from cross III were

backcrossed to strain NP1 and 16 pairs were isolated in each case. The results are shown in Table 1 (cross IV). None of the backcross progeny expressed the *ts vac*$^-$ phenotype. The tests for CHX-resistance gave the expected 1:1 ratio. A second set of backcrosses to strain NP1 were performed (cross V, Table 1) using CHX-resistant

Table 1. *Genetic analysis of non-vacuole forming mutant*

(In crosses I and II individual pairs were isolated, and their exconjugants were separately tested. In the III crosses, exconjugants which had completed normal conjugation in mass culture and become CHX-resistant were selected from among their non-conjugated parents by cycloheximide treatment: single surviving cells were isolated and tested. Early and late refers to phenotypic tests performed at about 15 and 50 fissions after conjugation, respectively. In crosses VI and V, pairs were isolated but exconjugants were not separated. Genotype symbols after strain names refer to the *Chx* allele.)

Cross	Immature progeny scored	Progeny: phenotype and numbers			
		ts phenotype (*vac*$^-$: *vac*$^+$)		CHX (resistant:sensitive)	
		Observed	Predicted*	Observed	Predicted
I. NP1(+/+) × CHX-F_3 (*Chx*-1/*Chx*-1)	6†	0:6	—	6:0	6:0
II. NP1(+/+) × CHX-SS (*Chx*-1/+)	17†	0:17	—	10:7	8·5:8·5
III. NP1(+/+) × CHX-SS (*Chx*-1/+)					
Cross 1					
Early	87	0:87	—	87:0	—
Late	58	0:58	—	58:0	—
Cross 2					
Early	237	0:237	—	327:0	—
Late	237	0:237	—	237:0	—
IV. F_1 (*Chx*-1/+) × NP1(+/+)	217	0:217	27:190	121:96	108·5:108·5
V. Cross IV progeny × NP1	47	1‡:46	6:41	—	—

* Assuming that homozygosity for a recessive allele determines the *ts vac*$^-$ phenotype and that NP1 has a heterozygous germ line but a fully assorted macronucleus, i.e., pure for functional *ts vac*$^-$ alleles. A 1:7 ratio is predicted for entries IV and V as only ½ of the pooled crosses could produce *ts vac*$^-$ progeny, in a 1:3 ratio to wild type.

† Exconjugant pairs. Both exconjugants were in every case alike for *vac* and CHX phenotype.

‡ Mating type III, same as strain NP1.

progeny from crosses IV. In three sets of 12 crosses each, only 47 immature progeny were produced, indicating a decline in progeny viability as inbreeding progressed. Only one of these progeny showed the *vac*$^-$ phenotype, but it turned out to be the same mating type at NP1; this 'progeny' is probably a non-conjugant NP1 cell, which gave a false negative maturity test. These results are inconsistent with the idea that the phenotype of strain NP1 is determined by a recessive mutation present also in its germ line, and suggest a macronuclear mutation.

The phenotype of NP1 has remained stable through several hundred fissions at 30 °C or room temperature. Somatic revertants of strain NP1 can be obtained, but only after mutagenic treatment (see Materials and Methods section). The revertants, selected for having regained the ability to divide at 37 °C, have also regained the ability to phagocytize normally at 37 °C. The induced frequency of somatic reversion is at least 4×10^{-7} mutants per mutagenized cell.

4. DISCUSSION

Three sites were initially considered as possible locations for the NP1 mutation: (1) *extra-nuclear sites*, involving either the transmission of cytoplasmic genes or cortical inheritance; (2) *micronuclear sites*, characterized by transmission of the trait through sexual reproduction; and (3) *macronuclear sites*, characterized by a loss of the trait during sexual reproduction. The phenotypical identity of exconjugants (crosses I and II, Table 1) rules out simple cytoplasmic or cortical inheritance. The failure of the *ts vac*$^-$ phenotype to appear among the 228 backcross progeny examined (crosses IV and V, Table 1) rules out a single micronuclear mutation. It is equally difficult to accept the idea of recessive mutant alleles at several unlinked genes, since this would make the probability of isolating the mutant very small in the first place. Consequently, we favour the idea that the mutation that determines the *ts vac*$^-$ phenotype of strain NP1 is a macronuclear mutation.

Macronuclear mutations in syngen 1 have been previously considered to determine mutant phenotypes in several instances (Nanney, 1959; McCoy, 1973; Byrne & Bruns, 1974). Conclusive evidence for the macronuclear location of somatic mutations to cycloheximide resistance in syngen 1 has recently been obtained (Orias & Newby, 1975). In view of the apparent 45-ploidy of the syngen 1 macronucleus (reviewed in Orias & Flacks, 1975) it may seem at first surprising that macronuclear mutants can be isolated. The difficulty is circumvented, however, because of a remarkable property of the syngen 1 macronucleus: the random distribution of daughter gene copies at the time of macronuclear division (Nanney, 1964; Orias & Flacks, 1975). This mechanism allows the chance build-up of mutant gene copies in some descendants of the original mutant cell, allowing expression of the mutant phenotype. The vegetative stability of strain NP1 and the failure to detect spontaneous somatic revertants strongly suggest that all the wild-type alleles were replaced by mutant alleles in the macronucleus of NP1 cells. This strain would thus be *functionally* analogous to a strain whose macronucleus was derived from a fertilization nucleus homozygous for the mutant allele(s) in question (with the exception that the trait is not transmissible to sexual descendants).

The mutational event(s) that gave rise to strain NP1 deserve closer scrutiny. Two alternatives can be considered, but no longer distinguished in the case of this mutant. (1) The mutation occurred after the first *post-zygotic* division of the fertilization nucleus during conjugation (the earlier the occurrence of the mutation during the development of the new macronucleus, the easier the replacement of

wild-type with mutant alleles). If the mutation was induced by the mutagen, one must assume that the modification of the DNA involved a sequence of chemical events initiated by the mutagen but not completed until at least three nuclear divisions later. (2) Alternatively, and in view of the fact that NP1 has mating type III, it is possible that NP1 is a vegetatively derived mutant of strain D1968–3. If so, then one must assume that the mutation is of spontaneous origin, since this parental strain was not mutagenized.

There is as yet no experimental basis for the genetic analysis of somatic mutations in *Tetrahymena*. Consequently, we cannot inquire into the number of mutations responsible for the phenotype of NP1, nor ask whether or not the mutation(s) that determine(s) temperature-sensitivity of growth and OA development are the same. The isolation of NG-induced revertants that have re-acquired the wild-type phenotype in both respects (growth and phagocytosis) suggests that both phenotypes are determined by the same mutation, but is not conclusive proof. The same general limitation prevents consideration of the relationship between the gene(s) mutated in NP1 and the gene responsible for slight abnormalities in the development of the OA in inbred strain D (Kaczanowski, personal communication), and which do not affect phagocytosis.

In view of the great utility of this mutant for various physiological and biochemical studies in tetrahymena cells (Ling; Silberstein; unpublished observations; Rasmussen, personal communication) it is unfortunate that the mutant phenotype of NP1 cannot be genetically combined with other mutations because, of the normal destruction of the old macronucleus during conjugation. There is no reason, however, why the same mutation could not be induced in the micronucleus. Further studies are in progress in our laboratory, using recent methods of mutant isolation (Orias & Bruns, 1974) which discriminate against macronuclear mutations.

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