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The first case of linkage in *Paramecium aurelia*

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1. INTRODUCTION

Although *Paramecium aurelia* has long been utilized for genetic studies, no genetic map has yet been established. At least two reasons are responsible for this situation. First, this species has a very high haploid number of chromosomes, probably around 40 (Dippell, 1954). Secondly, a relatively small number of genes are known (cf. Preer, 1968) because obtaining nutritional markers has not been practicable.

Recently, however, decisive progress has been made by the systematic search for thermosensitive mutations (Igarashi, 1966; J. Beisson, unpublished; T. M. Sonneborn, personal communication). Such mutations are readily inducible by various mutagenic agents (X-rays, ultraviolet, nitrosoguanidine) and they are easy to detect. Thermosensitive paramecia grow normally at usual or optimal temperatures (up to 28 °C) but die at higher temperatures (35–36 °C) at which wild-type cells survive and grow.

The data reported here indicate a close linkage between two thermosensitive mutations and define *P. aurelia's* first linkage group.

2. MATERIAL AND METHODS

The wild-type stock, from which all the mutants used in these experiments were obtained, originated from stock d4-2 (a non-killer strain with stock 51 genetic background) kindly supplied by Dr Sonneborn.

Four mutants were used: two thermosensitives obtained by ultraviolet irradiation, ts_{401} and ts_{111}; a morphological mutant, m_1, and a complex morphological and thermosensitive mutant, ts_{21m} obtained by nitrosoguanidine treatment. Each of these mutants is inherited as a single recessive gene. Aside from the four stocks homozygous for the listed mutations, we also used a double mutant stock, homozygous for genes m_1 and ts_{401}.

The mutants exhibit the following phenotypes:

Homozygotes for gene ts_{401} grow normally at 28 °C but die at 35–36 °C within 48 h after a few fissions. Before dying, they become swollen and yellowish.

Homozygotes for gene ts_{111} grow normally at 28 °C but die at 35–36 °C within 24 h after one or two fissions. Before dying they acquire a typical round shape, then burst or lyse.

Homozygotes for gene ts_{21m} display both slow growth at 28 °C (2–3 fissions per day instead of 4–5 for wild-type cells) and thermosensitivity. They die at 35–36 °C within about 36 h. Besides, they swim slightly slower and are larger than normal cells.

Homozygotes for gene m_1 are characterized by: (1) a very abnormal twisted shape, the anterior product of fission being less abnormal than the posterior product, (2) a slow growth (2–3 fissions per day at 28 °C), (3) a tendency to give monsters due to incomplete fissions and (4) abnormal trichocysts. (Two more apparently identical mutations have been obtained independently: one in our laboratory (m_2) and one by S. Pollack (personal communication).) The genetic relationship between these three are now under study by S. Pollack.

Double homozygotes for genes m_1 and ts_{401} are morphologically identical to single homozygotes for m_1 only; but, unlike m_1 homozygotes, they die at 36 °C.

Genes ts_{111} and m_1 are inherited independently of gene ts_{401}, as are more than 25 other mutations obtained in our laboratory (J. Beisson, unpublished).

Thermosensitivity tests. The thermosensitive characters are routinely assayed by the following procedure. Clones are tested by a duplicate transfer: one cell is placed at 28 °C, two cells at 36 °C. Both subcultures are examined on the following day. A thermoresistant clone always grows slightly faster at the higher temperature than at the lower one and this fact is in itself a reliable indication. Nevertheless, both subcultures were always re-examined the next day for a definitive classification.

Culture methods and genetic analysis. Cells were grown in Scotch grass bacterized with *Aerobacter aerogenes* according to the usual techniques (Sonneborn, 1950). Genetic analysis was performed as described by Sonneborn (1950). About 2 h after mixing the two populations to be crossed, tight conjugating pairs were isolated and after completion of conjugation, the two mates of each pair were isolated in separate slide depressions. Daily transfers (single cell re-isolations) were carried out at 28 °C with each clone developed from an ex-conjugant. Growth rate and phenotype were recorded daily and thermosensitivity tests were performed when the F_1 or F_2 clones were about 6–10 fissions old. F_2's were obtained from heterozygous F_1 clones by autogamy.

Autogamy, in syngen 4, occurs almost simultaneously in all the cells of a clone when the food gets exhausted, if the clones are over 25 fissions old (counting from the time of conjugation). The nuclear re-organization that takes place at autogamy renders each cell homozygous for all its genes. When autogamy occurs in a clone heterozygous for one pair of alleles, a 1:1 ratio of both homozygous genotypes is observed; if the clone was heterozygous for two pairs of unlinked genes, a 1:1:1:1 ratio for the four possible homozygous genotypes is obtained, etc....

Occurrence of autogamy was ascertained by observation of macronuclear breakdown in a stained (Dippell, 1955) sample of 50–100 cells out of a population of about 1000 cells. If macronuclear breakdown is observed in more than 98 % of the stained cells, the clone is classified as autogamous and single cells are isolated

from the rest of the population: they will produce the F_2 clones. Because of this procedure, it may happen that a few individuals, isolated from a supposedly 100 % autogamous population, have not undergone autogamy. If such individuals are isolated from a heterozygous population, they will of course still produce heterozygous clones.

3. RESULTS

(i) *Linkage of genes* ts_{401} *and* ts_{21m}

Linkage was observed when stock ts_{21m} (but not any other stock) was crossed with stock ts_{401}. In order to ascertain that the observed segregation was not due to any abnormal behaviour peculiar to gene ts_{21m}, stock ts_{21m} was also crossed to stock ts_{111}. The results are given in Tables 1 and 2.

Table 1. *Results of crosses between stock* ts_{21m} *(mating type VII) and stock* ts_{401} *(mating type VIII)*

Parents		F_1 phenotypes	F_2 phenotypes: +	ts_{401}	ts_{21}	Dead or undeterm.	Total no. of F_2 clones
1st cross (1 pair)	1A	+	0	60*		0	60
	1B	+	3	56*		1	60
2nd cross (1 pair)	2A	+, mt. VIII	1	48	63	8	120
	2B	+, mt. VII	7	56	55	2	120
3rd cross (3 pairs)	3A	+, mt. VIII	0	57	49	14	120
	3B	+, mt. VII	0	61	56	3	120
	4A	+, mt. VIII	3	49	61	7	120
	4B	+, mt. VII	2	61	44	13	120
	5A	+, mt. VIII	1	67	45	7	120
	5B	+, mt. VII	2	54	58	6	120
Total (2nd and 3rd cross)			16	443	431	60	960

* These figures represent the sum of both categories of thermosensitive clones (see text). +: normal morphology and growth rate and thermoresistance; ts_{401}: normal morphology and growth rate and thermosensitivity; ts_{21m}: phenotype ts_{21m}. The class of 'dead or undetermined' includes the wild type false 'homozygotes' (see text).

From each cross, 10–12 pairs were isolated and F_1 phenotypes determined for both clones of each pair. At least one pair was selected to carry out the F_2 analysis. The basis of selection was observation of wild-type characteristics in both F_1 clones of a pair, indicating that cross-fertilization had occurred.

In the first cross $ts_{21m} \times ts_{401}$ (top two lines, Table 1) F_2 clones were scored only as thermosensitive or thermoresistant, because this was the fastest way to check independence versus linkage between two thermosensitive mutations: any significant deviation from a 3:1 ratio for thermosensitive versus thermoresistant clones among F_2's would indicate linkage. In subsequent crosses, other aspects of the mutant phenotype (growth rate, cell size) were examined in order to

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distinguish ts_{21m} from ts_{401} or ts_{111} phenotypes. However, clones of double mutant genotype (ts_{21m}–ts_{401} or ts_{21m}–ts_{111}) could not be phenotypically distinguished from clones of genotype ts_{21m}.

It can be seen that while genes ts_{21m} and ts_{111} segregate independently (Table 2: 106 wild-type recombinants out of 420 F_2 clones), genes ts_{21m} and ts_{401} are closely linked: there are only 19 clones of thermoresistant phenotype out of 1020 classified F_2 clones (Table 1, including the first cross).

In order to be sure that this result meant linkage it was necessary: (1) to ascertain the genotype of the phenotypically wild-type F_2 clones, and (2) to identify double mutants of genotype ts_{21m}–ts_{401} among the F_2 clones.

Table 2. *Results of crosses between stock* ts_{21m} *(mating type VII) and stock* ts_{111} *(mating type VIII)*

Parents		F_1 phenotype	F_2 phenotypes: +	ts_{111}	ts_{21}	Dead or undeterm.	Total no. of F_2 clones
1st cross (1 pair)	1A	+, mt. VIII	37	35	74	4	150
	1B	+, mt. VII	37	35	75	3	150
2nd cross (2 pairs)	2A	+, mt. VIII	7	8	11	4	30
	2B	+, mt. VII	9	4	14	3	30
	3A	+, mt. VII	9	7	10	4	30
	3B	+, mt. VIII	7	8	11	4	30
Total			106	97	195	22	420

The symbols are the same as in Table 1. ts_{111}: normal morphology and growth rate, thermosensitivity.

(ii) *Genetic analysis of wild-type* F_2 *clones from crosses* $ts_{21m} \times ts_{401}$

As pointed out previously, phenotypically wild-type F_2 clones could be either true wild-type recombinants (homozygous for the wild-type alleles of genes ts_{21m} and ts_{401}) or clones derived from F_1 cells having escaped autogamy and remained heterozygous.

The simplest way to check the genotype of such clones was to induce autogamy and isolate single autogamous cells. Any still heterozygous clone would show a segregation of the different homozygous genotypes. On the other hand, any homozygous wild-type clone would give only wild-type cells. Furthermore, young clones originating from cells having escaped autogamy would still be competent for autogamy, while clones originating from autogamous cells would not be able to undergo autogamy before about 25 more fissions.

All the F_2 wild-type clones were examined in these two ways: (1) Most of them (all of the 19 listed in Table 1) were unable to undergo autogamy before completion of the normal 25 fissions and gave, after autogamy, homogeneous wild-type progenies (30 clones studied per progeny). (2) A few of the supposedly homozygous wild-type recombinants proved indeed to have been still heterozygous (and were therefore omitted from the + column, but entered in the 'dead or undetermined'

column of Table 1). They were found to undergo autogamy after only 6–10 fissions and after autogamy they produced mostly thermosensitive clones having either slow (ts_{21m}) or normal (ts_{401}) growth rate, and very few or no thermoresistant clones (+). The delayed F_2's obtained from two such false homozygous clones are given in Table 3. These two exautogamous populations are indistinguishable from the F_2's listed in Table 1.

Table 3. *Segregation after autogamy from two pseudo wild-type recombinants from crosses* $ts_{21m} \times ts_{401}$

Clone	F_1 phenotype	F_2 phenotypes: +	ts_{401}	ts_{21m}	Dead	Total no. of F_2 clones
2A9	+, mt. VIII	1	13	14	2	30
2B13	+, mt. VII	1	12	17	0	30

The symbols are the same as in Table 1.

(iii) *Identification of double mutants* ts_{21m}–ts_{401}

In order to identify clones of homozygous genotype ts_{21m}–ts_{401} among phenotypically ts_{21m} F_2 clones, these clones were crossed to homozygotes for gene ts_{401} and a morphological marker m_1. For obvious practical reasons, this study was not carried out for all 431 clones of phenotype ts_{21m} listed in Table 1, but was limited to the sample of 14 + 17 = 31 clones of phenotype ts_{21m} listed in Table 3.

(*a*) F_1 *analysis*. Since both parents (F_2. ts_{21m} and stock m_1 ts_{401}) were morphologically abnormal, every pair acquiring normal morphology and growth rate in the F_1 would necessarily be heterozygous and therefore have undergone cross-fertilization. For each of the 31 crosses, 10 pairs were isolated and all the pairs (at least three for each cross) showing morphological normality of the clones from both members of the pair were examined for thermosensitivity. Persistence of the thermosensitive character in all 6 (or more) clones indicated that the ts_{21m} parent was homozygous for gene ts_{401}. Acquisition of thermoresistance in all six (or more) F_1 clones could only be explained if the ts_{21m} parent did not carry gene ts_{401}.

In this way, two double mutants ts_{21m}. ts_{401} were identified in the offspring of clone 2A9 and one in the offspring of clone 2B13 (cf. Table 3). This result is quite consistent with the expected number of double mutants in these particular progenies, which is theoretically equal to the observed number of wild-type recombinants: one in each case (Table 3).

(*b*) F_2 *analysis*. Further confirmation of the double mutant genotype was obtained by studying F_2 segregation from two pairs of the preceding F_1 clones. The results are given in Table 4. All the F_2 clones were thermosensitive and 25% of them were normal for morphology and growth rate, indicating that genes ts_{21m} and m_1 are unlinked.

Table 4. *Results of crosses between double mutants* $ts_{21m} \cdot ts_{401}$ *and stock* $m_1\ ts_{401}$

Parents phenotype	F_1 phenotype	F_2 phenotypes				Total no. of F_2 clones
		ts_{401}	$m_1\ ts_{401}$	ts_{21m}	Dead	
6b $m_1\ ts_{401}$	ts_{401}	8	9	10	2	30
ts_{21}	ts_{401}	8	13	8	1	30
9c $m_1\ ts_{401}$	ts_{401}	7	10	10	3	30
ts_{21}	ts_{401}	7	14	9	0	30
Total		30	46	37	6	120

ts_{401}: normal morphology and growth rate, thermosensitivity. The other symbols indicate phenotypes identical to those of stocks homozygous for the corresponding genotypes.

4. DISCUSSION

As judged by the frequency of wild-type recombinants obtained in F_2's from crosses $ts_{21m} \times ts_{401}$, genes ts_{21m} and ts_{401} are undoubtedly closely linked. In the relatively small groups, the frequency of + recombinants varied from 0 to 6 %, possibly in part because of temperature differences during meiosis when samples were brought to room temperature for staining and isolation. The frequency of crossing over is probably less than 10 %.

Although our evidence for presence and frequency of the double mutants, ts_{21m}–ts_{401}, in F_2 populations, is based on analysis of only 60 F_2 clones (Table 3), and although complete genetic demonstration, including F_2 analysis, of the double mutants was obtained for two pairs only, the results presented here seem to suffice to support the conclusion that genes ts_{21m} and ts_{401} are linked. This is the first case of linkage in *Paramecium*.

SUMMARY

In *Paramecium aurelia*, syngen 4, gene ts_{21m}, which is shown to segregate normally and independently of two other genes: ts_{111} and m_1, is closely linked to gene ts_{401}. The frequency of their recombination is of the order of a few per cent. Genetic analysis was carried out to confirm the genotypes of both double mutants and wild-type recombinants among F_2 clones from crosses $ts_{21m} \times ts_{401}$. This is the first case of linkage so far reported in *P. aurelia*.

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