Genetic control of chemical induction of conjugation in *Paramecium bursaria*

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In six out of 36 natural stocks of *Paramecium bursaria* examined, chemical induction of conjugation was successful when cells were cultured in Ca-poor medium and then treated with acriflavine and KCl. Conjugation by the normal process following mixing of cells of opposite mating type occurred normally in these six stocks. Genetic analysis of chemical induction of conjugation was performed with three out of the six stocks. Chemical induction of conjugation was found to be controlled by recessive genes at two different loci, designated *cic-1*, *cic-2*. These genes are not linked to mating type loci. These inducible stocks showed additional features different from those of uninducible ones. When uninducible stocks were stimulated both by mating reaction and conjugation-inducing chemicals simultaneously, conjugation was drastically inhibited. Inducible stocks, by contrast, were free from such inhibition. The results suggest that there is a step blocked by the conjugation-inducing chemicals in the normal process of conjugation. This step is probably controlled by the *cic* loci.

INTRODUCTION

Conjugation in *Paramecium* can be induced in two different ways. One is normal conjugation induced by mixing complementary mating types. In normal conjugation, interaction of cells of complementary mating types activates the cells for pair formation. The other means of inducing conjugation is called the chemical induction of conjugation and was developed by Miyake (1958, 1968). In this type of conjugation, cells are activated for pair formation without the interaction of cells of complementary mating types.

Chemical induction of conjugation has made *Paramecium* a good model system for the study of activation in fertilisation. Another model system is that of artificial parthenogenesis in metazoan eggs (Metz, 1954; Hiwatashi, 1981, 1985). In particular, the discovery of mutants affecting chemical induction of conjugation has deepened the study of activation-initiating mechanisms (Cronkite, 1974, 1975). Furthermore, chemical induction of conjugation is useful for producing completely homozygous strains in species of *Paramecium* lacking natural autogamy. When conjugation is

induced chemically, aberrant cell unions often occur (Miyake, 1958). In these cell unions, exchange of gamete nuclei is blocked because of the failure of face-to-face adhesion of paroral regions and fusion of identical haploid nuclei occurs, leading to the production of completely homozygous progeny, like those found after autogamy (Miyake, 1968). Though many species of *Paramecium* have been shown to undergo chemically induced conjugation, this has not previously been reported for *Paramecium bursaria*. As will be shown in this paper, most natural stocks of this species cannot be induced to conjugate by chemical treatment. However, in this paper, I now report some stocks of *Paramecium bursaria* in which conjugation is chemically inducible. A genetic analysis of these stocks is also described.

MATERIALS AND METHODS

Stocks and culture methods

The stocks and strains used in this study belong to *Paramecium bursaria*, syngen 1. Stocks Nn7, T151, Mts5, d-492 and FF12 can be induced to conjugate by chemical treatment. Mts4 and T81C2 are uninducible stocks. Nn7, T151, Mts5 and Mts4

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are natural stocks and all others are strains derived from crosses or artificially induced cytogamy (Endoh, unpublished). T81C2 is a complete homozygote derived from the natural stock T81. This homozygous strain was obtained by artificial induction of cytogamy by a method similar to that of Orias *et al.* (1979) in *Tetrahymena*.

Culture medium used for the cultures was sterilised fresh lettuce juice inoculated with *Klebsiella pneumoniae* 1–2 days before use (Hiwatashi, 1968). Ca-poor lettuce juice medium in which the juice was diluted with 2 mM sodium phosphate buffer (pH 6.4) instead of Dryl's solution was used for culturing cells for chemical induction of conjugation. All cultures were kept at 25°C under the 16:8 (hour) light-dark cycle. All experiments were carried out at midday when mating reactivity was the strongest in the circadian cycle (Cohen, 1964).

Chemical induction of conjugation

Chemical induction of conjugation was carried out according to the methods of Miyake (1958, 1968). Mating reactive cells cultured in Ca-poor medium were washed twice and suspended in physiological balanced solution (PBS) containing 1.5 mM NaCl, 0.05 mM KCl, 0.1 mM MgCl₂ and 0.01 mM CaCl₂ in 2 mM phosphate buffer (pH 6.4). The cell suspension was mixed in a test tube with an equal volume of the conjugation-inducing medium (4–8 mM KCl, 20–80 µg/ml acriflavine, 100 mM acetamide in PBS). When successful, conjugation was induced in 2 hours in this medium (Endoh, 1987).

Crossing technique and identification of the phenotypes

When cells of complementary mating types are mixed, initial cell agglutination (mating reaction) occurs between complementary cells. In order to confirm conjugating pairs to be true cross pairs, cells were marked by the presence or absence of symbiotic algae in every cross breeding analysis, although selfing conjugation does not occur in *P. bursaria* under normal conjugation (Larison and Siegel, 1961; Endoh *et al.*, 1987). Six to ten hours after initiation of the mating reaction, conjugating pairs were isolated into depression slides containing Dryl's solution and kept in this solution. Three days later, fresh culture medium was added to each depression and cells were cultured in this medium for 3–4 days. After growth to 20–40 cells, a single cell from each depression was isolated (sub-caryonidal isolation) and cultured for another 3–4 more

days. Then almost all cells in each depression were transferred into a test tube. Established clones were tested for their mating reactivity. MR (macronuclear regeneration) clones which show high mating reactivity were discarded. True exconjugant clones would be in the period of immaturity and non-reactive. When clones became mature after successive transfers of culture, their capability to undergo chemical induction of conjugation was examined. Uninducible clones were cultured again and about 15 fissions (three tube transfers) after the first test, the test for chemical induction was repeated. In this way, the inducibility of each stock was tested at least three times.

RESULTS

Chemical induction of conjugation in natural stocks

Thirty-six natural stocks of *P. bursaria*, syngen 1, mainly collected in Japan, were examined for chemical induction of conjugation. Typical conjugants induced chemically are shown in fig. 1. As seen in table 1, six stocks among 36 examined were found to be chemically inducible. These six induc-

Figure 1 A typical conjugating union induced chemically. Stock: T151 (IV).

Table 1 *P. bursaria* stocks tested for their capability of chemical induction of conjugation

Stock	Mating type	Geographic origin	Chemical induction ^a of conjugation
Ih1	I	Iwate	—
Yt2	III	Yamagata	—
Yt3	III		—
Yt4	IV		—
M3	III	Miyagi	—
M4	II		—
M5	II		—
Tk1	I	Tochigi	—
T151	IV	Ibaraki	H
T157	II		—
T171	III		H
T172	II		—
T316	IV		—
T81	III		—
T81C2	III		—
Cw	III		—
Bg	I		—
Nn1	II	Niigata	L
Nn4	III		—
Nn5	IV		—
Nn6	II		—
Nn7	IV		H
Nn8	IV		H
So5	III	Nagano	—
So11	I		—
So13	II		—
So15	IV		—
Mts1	IV		—
Mts3	III		—
Mts4	I		—
Mts5	II		L
Ok1	I	Aichi	—
Ok2	III		—
Ok3	II		—
Cs2	II	Shanghai, China	—

^a H: Highly inducible; L: Barely inducible; —: Uninducible; See text in detail

ible stocks were collected from three different places and were divided into two groups by the extent of conjugation induced. One group consisting of Nn7, T151, Nn8 and T171 showed a high frequency of conjugation, usually more than 50 per cent. The second group consisting of Nn1 and Mts5 showed low frequency of conjugation, never above 10 per cent. These data show that in natural populations of *P. bursaria*, chemically uninducible stocks are common and widely distributed, while the inducible ones are relatively uncommon. This is just the opposite situation to *P. octaurelia* where the uninducible trait was mutant (Cronkite, 1974, 1975).

Genic control of the chemical induction of conjugation

Breeding analyses were carried out with the three stocks Nn7, T151 and Mts5. Nn1, Nn8 and T171 have not been genetically analysed because of their poor survival after conjugation. Table 2(a) shows the results of crossing together inducible and uninducible stocks, and the mating types of the progeny obtained. Mating types are determined by specific combination of pairs of alleles at two independently assorting loci. Mating type I is brought about by dominant alleles at loci *A* and *B*; type III is determined by the double recessive genotype

(*aabb*); type II (*aaBB* or *aaBb*) and IV (*AAbb* or *Aabb*) are determined by recessive alleles at one or the other of the two loci in combination with at least one dominant gene at other locus (Siegel and Larison, 1960, Siegel, 1963). When the original stocks Nn7 and T151 were used, high lethality was observed in F_2 and the segregation of mating type markers was much distorted. For this reason, the F_2 segregants, d-Nn7 and d-T151 which showed good fertility, used for the cross breeding analyses. When the chemical inducible strain d-Nn7 derived from Nn7 was crossed to the chemically uninducible T81C2, all F_1 clones showed the chemically uninducible phenotype. When F_2 progeny were obtained by a sibling cross of F_1 , uninducible and inducible clones segregated in a ratio not significantly different from 3:1. In order to classify the phenotypes, I repeated the test three to five times. The results suggest that the chemically inducible phenotype of stock Nn7 is controlled by a single recessive gene.

The same kind of genetic analysis was performed with another chemically inducible strain, d-T151 derived from T151. As seen in table 2(b), the results were almost the same as those of d-Nn7 and again the segregation ratio of the trait in F_1 and F_2 was consistent with a one gene hypothesis.

Chemical induction of conjugation in stock Mts5 was a little different from that of the former two stocks (d-Nn7 and d-T151). The chemically

induced conjugation was classified as "low" in Mts5, as shown in table 1. In this case, mating types are determined by only *B* locus because mating type II and III is recessive homozygous at *A* locus. When Mts5 was crossed to the uninducible stock, T81C2, the phenotypes of the progeny could not be allocated to two categories of inducible and uninducible (table 2(c)). About 10 per cent of F_1 (4/37) were inducible but the conjugation frequency induced was very low. When two F_1 clones with low inducibility were selected and sib-crossed to produce an F_2 , the segregation ratio of chemically uninducible to inducible trait was not significantly different from the expected ratio of 3:1 although segregation of mating types was not normal. Biased segregation ratio of mating types may be due to the close linkage between mating type gene and lethal gene. If I assume that dominance of uninducible character over inducible one is not complete and some heterozygotes show low inducibility, F_1 and F_2 segregations conform to simple Mendelian inheritance. To verify this hypothesis, F_1 clone with low inducibility was backcrossed to the original stocks Mts5 with low inducibility. As expected, uninducible plus barely inducible progeny to highly inducible progeny segregated 3:1. Segregation of mating type markers was also as expected. These results show that Mts5 is heterozygous for the gene controlling the chemical induction of conjugation, and that

Table 2 Breeding analyses of the trait for chemical induction of conjugation

	Cross	(Mating type : genotype)	Chemical induction of conjugation		Deviation from 1:3	Mating type				Deviation from 1:1:1:1
			Inducible	Uninducible		I	II	III	IV	
(a)	d-Nn7 ^a × T81C2	(I: <i>AaBb</i>) F ₁	0	45	—	13	10	12	10	0·8 < <i>P</i>
(b)	T81C2	(III: <i>aabb</i>) F ₂	19	63	0·7 < <i>P</i>	18	23	20	21	0·8 < <i>P</i>
	d-T151 ^b × T81C2	(I: <i>AaBb</i>) F ₁	0	39	—	10	9	11	9	0·95 < <i>P</i>
	T81C2	(III: <i>aabb</i>) F ₂	24	66	0·7 < <i>P</i>	20	22	22	26	0·8 < <i>P</i>

	Cross	(Mating type : genotype)	Chemical induction of conjugation			Deviation ^c from 1:3	Mating type		Deviation from 1:1
			Highly inducible	Barely inducible	Uninducible		II	III	
(c)	Mts5 ^c × T81C2	(II: <i>aaBb</i>) F ₁	0	4	33	—	17	20	0·6 < <i>P</i>
	T81C2	(III: <i>aabb</i>) F ₂	7	4	25	0·4 < <i>P</i>	26	10	<i>P</i> < 0·001
	F ₁ ^d × T81C2	(III: <i>aabb</i>) BC	11	1	26	0·4 < <i>P</i>	21	17	0·4 < <i>P</i>
	F ₁ ^d × Mts5	(II: <i>aaBb</i>)							
	Mts5	(II: <i>aaBb</i>)							

^a F_2 segregant from the cross Nn7 × Mts4. ^b F_2 segregant from the cross T151 × Mts4. ^c A natural stock (See table 1). ^d A segregant with barely inducible trait. ^e Assuming highly inducible clones are homozygous recessive.

Table 3 Complementation analyses among three conjugation inducible stocks

Cross (Mating type: genotype)	Chemical induction of conjugation		Mating type				Deviation from 1:1:1:1 or 3:1:1:3
	Inducible: uninducible		I	II	III	IV	
FF12 ^a × Nn7 (II: <i>aaBb</i>) (IV: <i>Aabb</i>)	53	0	15	12	13	13	0.9 < <i>P</i>
FF12 ^a × T151 (II: <i>aaBb</i>) (IV: <i>Aabb</i>)	0	41 ^b	9	12	8	12	0.7 < <i>P</i>
Nn7 × d-492 ^c (IV: <i>Aabb</i>) (I: <i>AaBb</i>)	0	94	35	12	10	37	0.9 < <i>P</i>

^a FF12 is an F₂ segregant with highly inducible trait derived from the cross Mts5 × T81C2 (See table 2(c)).

^b Segregants with barely inducible trait were counted as uninducible. ^c d-492 is an F₂ segregant derived from the cross d-T151 × Mts4 (See table 2(b)).

the dominance of uninducible character is not complete.

Complementation analyses

In order to determine the number of loci controlling the chemical induction of conjugation, complementation analyses were performed (table 3). In the complementation experiments, four different strains with high inducibility were used. Nn7 and T151 are natural stocks and homozygous for a gene controlling chemical induction of conjugation (table 2(a) and 2(b)). FF12 is a highly inducible segregant from the backcross in table 2(c). d-492 is a highly inducible F₂ segregant derived from T151 × Mts4. When FF12 was crossed to Nn7, all progeny were chemically inducible. This shows that inducibility of FF12 and Nn7 is controlled by the same locus. On the contrary, the remaining two crosses (Nn7 × d-492, FF12 × T151) produced uninducible progeny. These results suggest that the chemically inducible phenotype of T151 and d-492 (derived from T151) is controlled by a locus different from the one controlling FF12 and Nn7. Both genes were not linked to the mating type loci. The locus controlling Mts5 and Nn7 is designated as *cic-1*, and that controlling T151 as *cic-2*, indicating the first letters of the three words, chemical induction of conjugation.

Simultaneous stimulation

When cells were stimulated simultaneously by mating reaction and by conjugation-inducing chemicals, the induction of conjugation in uninducible stocks was very different from that in the inducible ones. When conjugation between uninducible stocks was induced by mating types in PBS, the conjugation ratio reached 50–85 per cent.

However, when conjugation between uninducible stocks was induced by mating types in conjugation-inducing chemicals, conjugation ratio decreased drastically (table 4). On the contrary, when conju-

Table 4 Inhibition of conjugation by simultaneous stimulation with mating reaction and conjugation-inducing chemicals

Cross	% conjugation	
	PBS ^a	CIC ^b
"Uninducible"		
So15 × Mts4	63.6	5.7
T316 × Yt5	64.6	11.5
Ok1 × Mts1	85.2	35.5
Ok1 × T316	50.4	1.8
Mts1 × Mts3	59.6	1.0
"Inducible"		
Nn7(<i>cic-1/cic-1</i>) × FF12(<i>cic-1/cic-1</i>)	83.4	88.9
T151(<i>cic-2/cic-2</i>) × 87-8 ^c (<i>cic-2/cic-2</i>)	63.7	75.2
FF12(<i>cic-1/cic-1</i>) × T151(<i>cic-2/cic-2</i>)	74.3	77.8

^a PBS: Physiological balanced solution. ^b CIC: Conjugation-inducing chemicals. ^c An F₂ segregant from the cross T151 × Mts4.

gation between the chemically inducible stocks was induced by mating reaction and conjugation-inducing chemicals, the ratio did not decrease but even increased slightly. In both cases, the initial mating reaction was not inhibited. In the uninducible stocks, the mating reaction in the conjugation-inducing chemicals continued at least 2 hours and thereafter most of the cell agglutinates were gradually broken up into single cells without releasing conjugating pairs. Such inhibition of the induction of conjugation never occurred when cells were homozygous for either of the *cic* genes. These results suggest that in the mating-type induced conjugation process, there is a step which is blocked by the conjugation-inducing chemicals.

DISCUSSION

Chemical induction of conjugation has previously not been successfully achieved in members of the "bursaria" group of *Paramecium* species, except for one selfing strain in *P. trichium* (Miyake, 1968). In the work reported here, 36 stocks were examined (table 1) for chemical inducibility of conjugation and it was found that only six of them could be induced to conjugate. Strains of *P. bursaria* capable of responding to chemical induction of conjugation appear to be distributed in widely scattered locations.

By genetic analysis, two loci controlling the susceptibility to chemical induction of conjugation were discovered. The susceptible stocks are recessive homozygotes of either of the two loci, *cic-1* and *cic-2*. When mating types of uninducible cells are mixed in the conjugation-inducing chemicals, strong mating reaction lasts for more than 2 hours but only very few pairs are induced. This inhibition of conjugation in mating type mixtures in the conjugation-inducing medium was not observed with the *cic* inducible stocks (table 4). Probably, the conjugation-inducing chemicals are acting on some steps of transition from mating reaction to pair formation and thus inhibiting the formation of pairs. In the *cic* mutants, this step which is susceptible to the conjugation-inducing chemicals is defective so that induction of pairs cannot be inhibited.

In *P. octaurelia*, Cronkite (1974) reported *kau* mutants which can be induced to conjugate by mating types but not by chemicals. There are two differences between the *kau* mutants in *P. octaurelia* and the *cic* inducible stocks in *P. bursaria*. Recessive mutations change cells from susceptible to non-susceptible to conjugation-inducing chemicals in the former species but from non-susceptible to susceptible in the latter. The other difference is the result of simultaneous stimulation by mating reaction and conjugation-inducing chemicals. The simultaneous stimulation inhibits conjugating pair formation both in inducible wild type and the uninducible mutants in *P. octaurelia* but only in uninducible stocks, not in the inducible ones in *P. bursaria*.

Kitamura (1982, 1984) reported that mating reactive cells of *Paramecium* tend to attach on hydrophobic surfaces such as the polystyrene surface and suggested that some kind of hydrophobic interactions on ciliary membranes is involved in the early step of the induction of conjugation. He hypothesized the possibility that the site for the hydrophobic interactions may be the receptor site

for chemical induction of conjugation. An interesting observation of Kitamura (1985) in relation to this study is that hydrophobicity of mating reactive ciliary surfaces of *P. bursaria* (uninducible stocks) appears very low comparing with that of other species of *Paramecium*. This fact may be in some way related to the low susceptibility of uninducible stocks of *P. bursaria* to conjugation-inducing chemicals. This possible relationship can be tested by comparing the hydrophobicity of mating reactive ciliary surfaces between uninducible stocks and the *cic* inducible stocks.

In *P. caudatum* and *P. octaurelia*, when chemical induction of conjugation was successful, cells settle to the bottom of containers and start to whirl a few minutes after cells are transferred into conjugation-inducing chemicals (Miyake, 1958; Cronkite, 1979; Kitamura and Hiwatashi, 1984). This inactivation of cell movement in conjugation-inducing chemicals never occurs when cells were in non-mating reactive stages or mutants unable to respond to the chemicals and thus gives us a clear prediction of successful induction of conjugation in those two species of *Paramecium*. Unexpectedly in *P. bursaria*, however, the situation was just the opposite. Cell movement of the *cic* inducible stocks was not inactivated in conjugation-inducing chemicals even though they were induced to conjugate chemically. On the contrary, chemically uninducible cells showed inactivation of cell movement in the chemicals (Endoh, unpublished). At present, I can find no good reason to explain these apparent contradictory phenomena.

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