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Source: Zoological Science, 12(2) : 219-223

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.219
Interspecific Pair Formation Induced by Natural Mating Reaction in Paramecium

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ABSTRACT—The species of Paramecium are sexually isolated primarily by the specificity of mating reaction. By this reason, it has been thought that interspecific pair formation does not occur when cells of different species are mixed. However we discovered that interspecific pair formation can be induced simply by mixing the mating reactive cells of two species. Among four species of Paramecium, P. tetraurelia, P. multimicronucleatum, P. caudatum, and P. bursaria, the interspecific pairs were observed in the former three species which belong to the “aurelia” group, but those three species did not mate with P. bursaria which belongs to the “bursaria” group. The percentage of interspecific pair formation was less than 10% in all positive cases. Macronuclear fragmentation, one of the remarkable nuclear changes in normal conjugation, was also observed in the interspecific pairs. The time course of the pair formation and macronuclear changes were similar to those of intraspecific conjugation.

INTRODUCTION

The process of conjugation in unicellular eukaryotic organisms is initiated by a species-specific cell adhesion as in fertilization of eggs with sperm in multicellular organisms. Species of Paramecium are classified into two large morphological groups: the “aurelia” group and the “bursaria” group. On the other hand, each taxonomical species of Paramecium is subdivided into many sibling species called syngens, the term coined by Sonneborn [27]. Syngen is reproductively isolated from each other by syngen-specific mating reaction [13–15, 17, 21, 24, 25]. In P. aurelia, however, all 14 syngens were later characterized biochemically and assigned species name [29]. In some species of Paramecium, such as P. caudatum and the P. aurelia complex, each syngen is composed of two complementary mating types, but other species such as P. bursaria, each syngen contains four or more complementary mating types. In P. caudatum and P. aurelia complex, the expression of mating types are potentially controlled by a pair of alleles with simple dominance. Genetic crosses have revealed that the dominant allele permits expression of the E type, while the recessive allele restricts homozygotes to the O type [12, 28]. In P. caudatum, by intersyngenic cross-breeding analyses Tsukii and Hiwatashi [32] found that three loci, Mt, MA, and MB are involved in the determination of syngen specific mating types.

When the mating reactive cells of complementary mating-types are brought together, they interact with the cilia located on the ventral surface of a cell (mating reaction) and form large agglutinates, called the mating clumps [2, 4, 11]. About 30 min after the formation of mating clumps ciliary degeneration begins from the anterior ventral surface of the cell [19, 22, 35]. Approximately one hour after the mating clump formation the holdfast union, which is a pair of cells united at their anterior region, is formed. In the holdfast union pairs, cells adhere at the surfaces of cell bodies where cilia degenerate. The following step of cell adhesion called the paroral union comes about 2 hr after the holdfast union formation. In the paroral union pairs, cells adhere at cilia-free surfaces of the region of the cytostome. Metz and his colleagues [17] have demonstrated that the series of events in the conjugation of Paramecium is activated by the initial mating type-specific adhesion of cells. However, Hiwatashi [10] has found that the formation of holdfast unions are not to be strictly mating-type specific, because the selling pairs of the same mating type are also observed after mating reaction.

Not only mating-type non-specific but also species-nonspecific conjugations are induced in chemical induction of conjugation. The chemical induction of conjugation has been demonstrated by changing the chemical composition of culture medium in various species of Paramecium [5, 8, 18, 22]. When two species of Paramecium belonging to the “aurelia” group are mixed, both intraspecific mating pairs and interspecific ones are usually formed by the chemical induction of conjugation method. This indicates that chemical induction of conjugation is species-nonspecific. In addition, Endoh [7] has demonstrated that interspecific pair formation can be induced between the cells belonging to the two different morphological groups, the “aurelia” and the “bursaria” groups. However it is unknown whether interspecific mating pairs are formed in natural mating reaction or not. In this report we will demonstrate that interspecific pair formation can be induced simply by mixing cells of complementary mating-types. In interspecific pairs, macronuclear fragmentation, such as occurs in normal conjugation and in cytogamy (autogamy in paired cells), is observed.
MATERIALS AND METHODS

Stocks and cell culture

The strains used in this study were 27aG3 (O³), C103 (E³) and 16B909 (E³) in Paramaecium caudatum, 53 (O²) and 49B (E²) in P. multimicronucleatum, 51S (O) and 51S (E) in P. tetrarreta, and I (O) and II (E) in P. bursaria. 27aG3 (O³) and C103 (E³) are wild-type in behavior and trichocyst non-discharge (TND) in exocytosis [31]. 16B909 is a double-mutant, caudatum non-reversal (CRN) in behavior [30] and TND in exocytosis.

Cells were cultured at 25°C in 1.25% (w/v) lettuce juice medium in K-DS which is the modified Dylene solution substituted KH2PO4 for NaH2PO4 [6] and inoculated with Klebsiella pneumoniae one day before use [12].

Mating reaction and interspecific mating pairs

Cells were cultured by inoculating several hundred cells into 2 ml of culture medium, adding 4, 8, and 10 ml of fresh culture medium on every successive days. The mating reactive cells were obtained at one or two days after the final feeding.

Two species of complementary mating-type cells, approximately 2500 cells each, were mixed. About 3 hr after the mixing, interspecific pairs were counted. To distinguish interspecific pairs from intraspecific ones the trichocyst non-discharge mutants, TND, were used in P. caudatum [31]. Interspecific pairs between P. multimicronucleatum and P. tetrarreta were identified by the difference of cell size. Because the cytoplasm of P. bursaria is green, it is easy to identify interspecific pairs between this species and the other ones. The conjugation specific nuclear process was examined by staining with the phenol-fuchsin method [3].

RESULTS

Interspecific pair formation within the “aurelia” group

When the mating reactive cells of four complementary mating-types belonging to two species were mixed, small mating clumps consisted of several cells of complementary mating-types in each species were formed in the beginning. Then, mating clumps of the both species were agglutinated together and large mating clumps were formed. About one hour after the mixing, three types of conjugating pairs, intraspecific in each species and interspecific, were observed. Since the cells of P. caudatum were TND mutants and the cells of P. multimicronucleatum were wild-type, interspecific mating pairs between P. caudatum and P. multimicronucleatum were identified by testing the ability to discharge trichocysts. The percentage of interspecific mating pairs induced in the “aurelia” group were summarized in Table 1. In case of the pair formation between P. caudatum and P. tetrarreta, interspecific mating pairs was 6.8% among 1471 mating pairs and 5.2% among 1315 mating pairs. Three combinations among three species were examined twice. In each experiment, the percentage of interspecific mating pairs was also examined to test mating reactivity and the activity of pair formation in each species. The percentage of intraspecific mating pairs in these experiments were estimated as 62.1 (Exp. 1) and 82.8 (Exp. 2) in P. caudatum, 31.1 (Exp. 1) and 12.0 (Exp. 2) in P. tetrarreta, and 69.9 (Exp. 1) and 42.6 (Exp. 2) in P. multimicronucleatum. Although the activity of intraspecific pair formation was relatively low in P. tetrarreta, the cells of P. tetrarreta mated with both P. caudatum and P. multimicronucleatum.

In natural mating reaction in P. caudatum, it is known that homotypic pairs, such as E-E or O-O, are also formed, albeit at a low percentage [10]. This and other findings suggest that the initial contact with cilia between the cells of complementary mating-type is mating-type specific but the formation of holdfast union is not mating-type specific [10, 20, 32]. To know whether the formation of interspecific mating pair is associated with the same mechanism postulated in the formation of intraspecific holdfast union, we compared the percentage of homotypic pairs of P. caudatum with that of interspecific mating pairs by using behavioral genetic

<table>
<thead>
<tr>
<th>Table 1. Interspecific mating pairs induced by natural mating reaction within the “aurelia” group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>P. caudatum</td>
</tr>
<tr>
<td>P. tetrarreta</td>
</tr>
<tr>
<td>P. multimicronucleatum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>Interspecific mating pairs (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P. caudatum</td>
<td>23.1**</td>
<td>76.4</td>
</tr>
<tr>
<td>P. tetrarreta</td>
<td>11.9</td>
<td>11.1</td>
</tr>
<tr>
<td>P. multimicronucleatum</td>
<td>64.6</td>
<td>58.8</td>
</tr>
</tbody>
</table>

* Numerals in parentheses represent the number of total pairs including both inter- and intraspecific mating pairs of each species. ** Mating reaction in each species was induced by mixing with approximately 2500 cells of each complementary mating type.

Table 2. Heterotypic and homotypic mating pairs of P. caudatum induced by natural mating reaction

<table>
<thead>
<tr>
<th>Combination of mating types</th>
<th>Mating pairs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-O</td>
<td>7.7</td>
</tr>
<tr>
<td>E-E</td>
<td>7.6</td>
</tr>
<tr>
<td>E-O</td>
<td>84.7</td>
</tr>
</tbody>
</table>

Mating reactive cells of 16B909 (E³, CNR) and 27aG3 (O³, wild-type) were mixed and stood at 25°C for 3 hr. The combination of mating types was identified by testing the ability to swim backward in the test solution (20 mM KCl in K-DS). Total pairs counted was 712. O and E indicate mating types O and E.
Interspecific mating pairs in *Paramecium*

The percentages of interspecific mating pairs were in the range of 2.0–8.2 while the percentage of homotypic pairs of *P. caudatum* was 15.3 (Table 1 and Table 2).

**Interspecific pair formation between species of the “aurelia” group and *P. bursaria***

When the mating reactive cells of *P. bursaria* were mixed with the mating reactive cells of *P. caudatum, P. tetraurelia,* or *P. multimicronucleatum,* the mating reaction of complementary mating-type cells and small mating clump formation within each species were observed in the same manner as interspecific mixture of “aurelia” group. Then, mating clumps of both white (*P. caudatum, P. tetraurelia,* or *P. multimicronucleatum*) and green (*P. bursaria*) cells were agglutinated together and large mosaic mating clumps were formed. However, mating pairs of *P. bursaria* with other *Paramecium* species were not observed at all (Table 3). The activity of interspecific pair formation in *P. multimicronucleatum* was 96.0% but no interspecific mating pair was observed among 1830 mating pairs.

**Nuclear process in interspecific mating pairs***

In the interspecific mating pair, the cells of two species adhered with each other at the anterior regions of the cell

<table>
<thead>
<tr>
<th>Combination</th>
<th>Interspecific mating pairs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. caudatum</em> + <em>P. bursaria</em></td>
<td>0 (626)*</td>
</tr>
<tr>
<td><em>P. tetraurelia</em> + <em>P. bursaria</em></td>
<td>0 (604)</td>
</tr>
<tr>
<td><em>P. multimicronucleatum</em> + <em>P. bursaria</em></td>
<td>0 (1830)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>Intraspecific mating pairs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. bursaria</em></td>
<td>14.1**</td>
</tr>
<tr>
<td><em>P. caudatum</em></td>
<td>16.7</td>
</tr>
<tr>
<td><em>P. tetraurelia</em></td>
<td>12.8</td>
</tr>
<tr>
<td><em>P. multimicronucleatum</em></td>
<td>96.0</td>
</tr>
</tbody>
</table>

* Numerals in parentheses represent the total number of pairs including intraspecific ones of each species.
** Mating reaction in each species was induced by mixing with approximately 2500 cells of each complementary mating type.

**Fig. 1.** Photomicrographs of interspecific pair formation and macronuclear fragmentation. A: Interspecific mating pair between *P. multimicronucleatum* (large cell) and *P. tetraurelia* (small cell). Scale, 50 μm. B: Macronuclear fragmentation at 20 hr after induction in *P. tetraurelia*. Scale, 25 μm. C: Macronuclear fragmentation at 20 hr after induction in *P. caudatum*. 50 μm. D: Macronuclear fragmentation at 20 hr after induction in *P. multimicronucleatum*. Scale, 50 μm.
bodies, just like holdfast unions in normal conjugation (Fig. 1 A). However in case of the mating pairs formed between _P. tetruraelia_ and other species, usually tight pairs, designated as paroral unions in normal conjugation, were not formed, although the cell contact continued for 7–9 hr. On the other hand, the mating pairs of _P. caudatum_ and _P. multimicronucleatum_ continued for 13–15 hr by forming tight pairs. We tested for macronuclear fragmentation in interspecific pairs by the phenol-fuchsin staining. In all combinations of interspecific conjugation, macronuclear fragmentation was observed (Fig. 1 B, C, D). This suggests that the interspecific mating pairs undergo self-fertilization, a process called cytogamy, though detailed observation of the behavior of micronuclei is necessary to prove it.

**DISCUSSION**

Since the discovery of mating types in _P. aurelia_ by Sonneborn [24], “species problems” in _Paramecium_ has been extensively studied by many investigators including inter-syngenic or interspecific matings in natural mating reaction [1, 9, 26, 28, 32]. The finding of interspecific pair formation induced by natural mating reaction described here brings up additional important problems both in physiological and evolutionary aspects of sexual isolation in ciliates.

The process of interspecific pair formation can be divided in three steps; In the first step, mating reaction occurs in each species forming small mating clumps of a single species. In the second step, the small mating clumps of both species aggregate together and eventually forms large mosaic clumps. In the third step, cells adhere to form holdfast unions. The first and the second steps were observed in not only when two species belonging to the “aurelia” group were mixed but also when _P. bursaria_ was mixed with species belonging to the “aurelia” group. The time course of the appearance of interspecific pair was similar to that of intraspecific ones in all cases tested. In addition, there are apparent similarities between the interspecific conjugating pairs and intraspecific homotypic selfing pairs: they are (1) nearly the same (usually less than 10%) percentage of pair-formation and (2) start of pairing at the anterior region of the cell body. These results suggest that a common mechanism is involved in the formation of interspecific pair formation and the formation of homotypic conjugating pair induced in mating reaction. Probably, the formation of mating pair is a non-specific random event occurring by chance at the cilia-free anterior region of cells. The probability of head-to-head contact may depend on the size of the cilia-free area. Thus, if mating clumps are composed of two species, it is possible to form holdfast unions with the cells of different species. However, when the mating reactive cells of complementary mating types in _P. bursaria_ were mixed with those of _P. caudatum_, although large-mosaic mating clumps were formed, no interspecific pairs were observed. This indicates that on the cell surface there are some specific substances associated with the formation of holdfast unions. These substances, called the “holdfast substances” [17], should be functional among the species belonging to the “aurelia” group but not be functional between species of the “aurelia” group and _P. bursaria_.

One alternative interpretation of the interspecific pair formation between _P. bursaria_ and species of the “aurelia” group is that the area of the cilia-free anterior region of _P. bursaria_ cells is too small to keep head-to-head contact with the species belonging to the “aurelia” group. Endoh [7] suggested that the size of the cilia-free area in _P. bursaria_ is not enough to form homotypic mating pairs in natural mating reaction.

Interspecific pair formation can be induced by chemical treatments among different species of the “aurelia” group [20] and between _P. bursaria_ and the species belonging to the “aurelia” group [7]. Endoh [7] found that the strain which conjugates with the cells of “aurelia” group by the chemical-induction of conjugation method was the mutant which was chemically inducible of conjugation and was found in natural stocks. There are some differences in interspecific pair formation between chemically-induced and mating-reaction-induced ones: (1) pairing is sometimes irregular in chemically-induced pairs, but is mostly head-to-head unions in mating-reaction-induced ones; (2) the cell agglutination comparable to the mating reaction is not required to induce holdfast union in the former, but interspecific mating agglutination is required for the pair formation in the latter. The molecular mechanisms of the chemical induction of conjugation is not fully understood yet, though Kitamura [16] suggested that cationic exchange on the cell surface is involved in the induction. It would be very interesting to know whether the mutant of _P. bursaria_ which can conjugate with the cells of “aurelia” group by the chemical-induction method can form interspecific pairs with the species of “aurelia” group in natural mating reaction.

There are significant differences between paramecia of the “aurelia” and the “bursaria” groups: 1) morphology [36], 2) the specificity of immaturin [23], and 3) patterns of hemoglobin polymorphism [33, 34]. As described in this article, the specificity of the formation of holdfast unions induced by natural mating reaction was also the group-specific. The analysis of molecular feature and localization of the holdfast substances would be important to understand the molecular mechanism of group specificity underlying between “aurelia” and “bursaria” groups.

Recently we found thousands of _Paramecium_ living in a small pond located on the east coast of Miyagi prefecture, Japan. There are many species including _P. caudatum_, _P. aurelia_ complex, and _P. multimicronucleatum_. Under an appropriate condition, they could contact during mating reaction and could form interspecific mating pairs in nature. It would be of interest to look for interspecific mating pairs in nature.

**ACKNOWLEDGMENTS**

We thank Dr. K. Hiwatashi for helpful comments on the
REFERENCES