Cellular Interactions during the Mating Process in *Chlamydomonas eugametos* 

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**ABSTRACT**

A method to determine the mating competence of *Chlamydomonas eugametos* was developed. The contribution of each mating type in the pair formation was investigated using asymmetric gamete mixtures. It was established that pair formation is not mediated by a pheromonal attraction mechanism between partner gametes, but depends on collision chances. On the other hand, it was demonstrated that during transient contacts between partner gametes the flagellar agglutinability of both partners is stimulated, evidently to prepare a successful mating. The *plus* mating type was generally less agglutinable than the *minus* mating type and was a rate-limiting factor in the mating process.

*Chlamydomonas eugametos* is a biflagellate unicellular green alga, reproducing vegetatively by sporulation. Its cell division can be synchronized by applying a light/dark regime on static liquid cultures (1). Upon nutrient deficiency the cells differentiate into gametes (19). *C. eugametos* is heterothallic and isogamous, which means that there are no morphological differences between gametes of the two mating types *mt*+ and *mt*-. After mixing, *mt*+ and *mt*- gametes agglutinate with their flagella, often in clumps of many cells. This sexual agglutination reaction is specifically characterized by the jerking movements of the gametes and is well distinguished from other accumulation phenomena like photo-, geo- and aerotaxis. By rearranging the flagellar contacts, the apices of two agglutinating cells of opposite mating type attain a position which enables fusion into a vis-à-vis pair. Vis-à-vis pairs swim around for several hours until they settle and develop into zygotes.

The recognition of partner cells takes place via the flagella. From flagella of *mt*+ gametes the glycoprotein responsible for the recognition and agglutination has been isolated and identified (7, 14). A similar molecule has been found on the *mt*+ flagella (8).

The question is whether contacts between gametes of opposite mating type occur via random collisions or whether a pheromonal attraction mechanism is involved. As has been reviewed by Wiese (21), chemotaxis between gametes of *C. eugametos* has been reported by Moewus in 1938, but others could not confirm this. However, Mesland (12) inferred—and due reserve—from his experiments that *mt*+ gametes of *C. eugametos* show a chemotactic response towards *mt*− gametes. With respect to isogamous systems in general, most authors argue that if pheromonal gamete attraction occurs, it will not develop into a genetically stable property unless morphological anisogamy or at least behavioral anisogamy (viz. motility dimorphism) co-evolve (5, 6, 9, 21, 22). *C. moewusii* var. rotunda is a well-known exception to this reasoning because it shows chemotaxis between equally motile isogametes. However, the substances produced by the attracting *minus* mating type also attract interspecifically both *mt*+ and *mt*- gametes of *C. eugametos* (20).

In this paper we reinvestigated the mating process in *C. eugametos*, using different approaches. Our conclusion is that chemotaxis is not involved in the mating of *C. eugametos*, but that partner gametes meet via random collisions. We demonstrate that a successful pair formation is prepared by transient contacts, during which the flagellar agglutinability of both partners is stimulated.

**MATERIALS AND METHODS**

**Cell Cultures.** *Chlamydomonas eugametos* strains UTEX 9 (mating type *plus*, *mt*+) and UTEX 10 (mating type *minus*, *mt*−) from the Algal Collection at the University of Texas (Austin) were grown in Petri dishes on an agar-containing medium on a 12 h light/12 h dark regime as described by Mesland (11). Gamete suspensions were obtained by flooding 2-week-old cultures with 20 ml distilled H2O just before the start of the dark period; the gametes were harvested at the beginning of the following light period. To determine cell densities, samples fixed with glutaraldehyde (final concentration 1.25% v/v) were counted in a haemocytometer. Gamete suspensions were diluted with cell-free medium. This was obtained by centrifuging a parallel batch of gamete suspension and filtering the supernatant through a 0.8 µm Millipore filter.

**Determination of Pair Formation and Mating Competence.** To determine pair formation in a mixture of the two mating types, the mixture was fixed with glutaraldehyde (final concentration 1.25% v/v) which disrupted agglutinating cells but not vis-à-vis pairs. Free cells and pairs were counted in a haemocytometer and the percentage of paired cells was calculated:

\[(2 \times \text{pairs } \text{ml}^{-1}) \times 100/(2 \times \text{pairs } \text{ml}^{-1} + \text{free cells } \text{ml}^{-1})\]

Mating competence is defined as the percentage of cells of a *mt*+ or *mt*− culture able to form vis-à-vis pairs. To establish this we developed a standard procedure. Details of this test are dealt with under "Results." The standard procedure to determine the mating competence of a suspension of, for instance, *mt*− cells was: *mt*− cells were mixed with an excess of *mt*+ gametes, usually in a ratio of 10:90 or 20:80 (with the same results), always at a total cell density of at least 2 × 10⁶ cells ml⁻¹ and placed under white fluorescent tubes (5500 lux, 65 µE s⁻¹ m⁻²). After 60 min the mixtures were fixed and counted. The mating competence of *mt*− was calculated as follows:

\[(\text{pairs } \text{ml}^{-1}) \times 100/(\text{mt}^- \text{cells } \text{ml}^{-1})\]

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To be sure that the $mt^+$ suspension used in this test was mating competent for at least 20%, it was always cross-checked as the minor partner against $mt^-$ gametes. Of all gamete suspensions used in the experiments described in this paper, we determined the mating competence in this way. All other determinations of pair formation by using different cell densities or time periods were carried out under the same conditions. All tests were made in triplicate.

**Assay for Agglutinability.** To determine the degree of agglutination, $mt^+$ and $mt^-$ gametes were mixed at a total cell density of $5 \times 10^6$ cells ml$^{-1}$. At zero time a sample of this mixture was transferred to a slide and examined by light microscopy. To determine the agglutinability of dead cells, gametes were fixed with glutaraldehyde for 2 min at 0°C, and twice washed with cell-free medium before being mixed with living partner gametes. Agglutination was followed with a Zeiss photomicroscope.

To measure the agglutinability in a semiquantitative way, a suspension of gametes of one mating type was fixed, washed, and then diluted in a binary dilution series. Each dilution was tested with a constant amount of living partner gametes. Agglutinability was expressed as the titer of the last dilution still showing agglutination. When the agglutinability was $2^*$, this means that the fixed gamete suspension reacted positively up to the fourth 1:1 dilution.

**RESULTS**

**Agglutination and Pair Formation in 50:50 Mixtures.** When $mt^+$ and $mt^-$ gametes were mixed in equal amounts, the size of clumps of agglutinating cells and the rate of pair formation were proportional to the cell density. At low cell densities (below $2 \times 10^6$ cells ml$^{-1}$) few, if any, clumps of 2 to 3 cells were formed and pair formation was slow. At high cell densities (above $2 \times 10^6$ cells ml$^{-1}$) clumps were large and, as shown in Figure 1, pair formation was completed after 60 min. Although the mating process had then come to an end, only 80% of the cells was paired. The remaining free cells could include mating competent cells unable to find a mating competent partner as well as mating incompetent cells. Therefore, pair formation of a 50:50 mixture gives no direct information about the mating competence of either partner.

**Pair Formation in Asymmetric Mixtures.** To determine pair formation in asymmetric mixtures, $mt^+$ and $mt^-$ gamete suspensions of $2.5 \times 10^6$ cells ml$^{-1}$ were used. These were mixed in different proportions, resulting in a series of suspensions with different mating-type ratios but with equal total cell densities. After a variable period of time, mixtures were fixed and counted for free cells and pairs. Figure 2 shows that a triangle is formed when pair formation is plotted against mating-type ratio, implying a direct proportionality between these two. In Figure 2a maximum pair formation was reached within 30 min, but only in the asymmetric mixtures. This indicates that finding a competent partner is easier with an excess of partner cells than with a more equal amount of them. After 150 min, pair formation was completed in all mixtures, resulting in a perfect triangle. In the triangle of maximum pair formation, the mating competence of each mating type can be found graphically, as shown in Figure 2: the part of the perpendicular erected in the middle of the triangle of maximum pair formation, the mating competence of the minor partner can be read: a, Pair formation after 30 min ($\circ\ldots\circ$) and 150 min ($\bullet\ldots\bullet$); mating competence of both $mt^+$ and $mt^-$ is 95%. b, Pair formation after 60 min; mating competence of $mt^+$ is 58% while the $mt^-$ suspension is mating competent for only 6%.

![Figure 1](https://www.plantphysiol.org/download/fig_1.png)

**FIG. 1.** Pair formation (percentage of paired cells) at variable cell densities in a mixture with equal amounts of $mt^+$ and $mt^-$ gametes after different time intervals. (A) 30 min, (○) 60 min, (■) 120 min.

![Figure 2](https://www.plantphysiol.org/download/fig_2.png)

**FIG. 2.** Pair formation (percentage of paired cells) in mixtures with different proportions of $mt^+$ and $mt^-$ gametes. Two experiments are shown (a and b). The total cell density in all mixtures was $2 \times 10^6$ cells ml$^{-1}$. Where the curves intersect the perpendicular of the base line of the triangle of maximum pair formation, the mating competence of the minor partner can be read. a, Pair formation after 30 min ($\circ\ldots\circ$) and 150 min ($\bullet\ldots\bullet$); mating competence of both $mt^+$ and $mt^-$ is 95%. b, Pair formation after 60 min; mating competence of $mt^+$ is 58% while the $mt^-$ suspension is mating competent for only 6%.
Mutual Stimulation of Agglutinability. The finding that the relatively weakly agglutinating \( mt^+ \) gametes were able to pair at a higher rate when confronted with an excess of partner gametes than the better agglutinating \( mt^+ \) gametes, as shown in Figure 3, suggested a stimulation of the agglutinability of \( mt^+ \) gametes by contacts with \( mt^- \) gametes. To test this possibility, \( mt^+ \) and \( mt^- \) gametes were mixed in equal amounts and at various time intervals samples were fixed, washed, and diluted as described in "Materials and Methods." Aliquots were mixed with living \( mt^+ \) and \( mt^- \) gametes respectively, to examine the agglutinability of the fixed \( mt^+ \) and \( mt^- \) flagella. The results of one typical experiment are given in Table II. Six experiments were done, all showing a clear mutual stimulation of the agglutinability of both partners during the mating process. In most experiments \( mt^- \) had a low agglutinability compared with \( mt^+ \) at zero time and although \( mt^- \) gametes showed a more distinct stimulation after mixing with partner gametes, they did not equal \( mt^+ \). This agrees with the difference in clump formation described above.

Our results lead to the following conclusions. \( Mt^+ \) gametes form smaller clumps with an excess of partner gametes than \( mt^- \) gametes, probably because \( mt^- \) flagella carry less agglutination sites than \( mt^+ \) flagella. Upon physical contact, the agglutinability of both partners is stimulated. Frequent contacts with partner gametes are required to raise the agglutinability to such an extent that the two partner cells can properly adhere and fuse. The poor agglutinability of \( mt^+ \) is a rate-limiting factor in the mating process, since it takes more time to stimulate \( mt^+ \) to the appropriate level of agglutinability than \( mt^- \). In a 10% \( mt^- \) mixture, \( mt^+ \) cells are sooner able to fuse than in a 10% \( mt^- \) mixture, because in the first mixture each of them collides more frequently with \( mt^- \) gametes. This is most clear at very low cell densities where clumping hardly occurs and pair formation is slow. Microscopical observation of a 50:50 mixture of low density (2 \( \times 10^5 \) cells ml\(^{-1}\)) confirmed the occurrence of transient contacts: two partner cells often agglutinated with only one flagellum each, wildly beating the free flagellum but eventually they lost contact and each cell resumed swimming in evidently random directions. These unstable contacts again underline the absence of any attraction mechanism.

**DISCUSSION**

A pheromonal attraction mechanism in unicellular organisms usually implies that the attracting partner has a low motility with respect to the attracted partner (13, 17, 21, 22). For *C. eugametos* it has been established that the swimming speed of \( mt^+ \) and \( mt^- \) gametes is identical, both before and after mixing (12). Chemotaxis to nutrient sources is not uncommon in *Chlamydomonas* (4, 18) but, using a variety of assays, we have not been able to detect consistent attractive actions of gametes *C. eugametos* to the opposite mating type. On the other hand, Mesland (12) demonstrated that \( mt^- \) gametes bind more \( mt^+ \) gametes than
Table II. Titer of Agglutinability of mt* and mt− Gametes after Mixing Both Mating Types in Equal Amounts at a Total Cell Density of 5 x 10^6 Cells ml⁻¹

Four parallel mixtures were made and every 5 min a mixture was fixed. The fixed mixtures were then washed and diluted in a binary dilution series. Aliquots of all dilutions were tested with living mt* and mt− gametes respectively. The last dilution of a series still showing agglutination expressed the titer of the agglutinability.

<table>
<thead>
<tr>
<th>Time after Mixing Living Gametes</th>
<th>Titer of Agglutinability after Fixation</th>
<th>mt* Gametes</th>
<th>mt− Gametes</th>
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<tr>
<td>min</td>
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Table II shows that mt− gametes excrete an attracting substance. In this paper we confirmed his finding, but we conclude that chemotaxis could not be the underlying mechanism, since even fixed mt− cells bind more mt* gametes than vice versa.

The difference in agglutinability between mt* and mt− proved to be a specific but accidental property of the two clones we—and Mesland (12)—used. Preliminary experiments with recombinant clones showed that the difference is not a rule and is certainly not sex-linked. However, this accidental asynchrony of the system gave us the opportunity to study whether, and how, the two mating types influence each other during the mating process.

Our explanation of the results is that repeated collisions with partner cells are needed before a given cell can establish a stable adhesion. Every encounter with a partner cell leads to an increase of agglutinability, so that, as the result of several collisions, two partner cells properly adhere and can fuse. Both the density and ratio of a mixture determine the chance that two partner cells meet and can stimulate each other. Since both parameters are the same in 10% mt* mixtures and in 10% mt− mixtures and since the system is strictly isomorphic, the observed difference in rate of pair formation cannot be explained from the geometry of the mixtures. The numbers of compatible collisions in both mixtures may be the same, their stimulating effects evidently are different. We assume that this is caused by the fact that mt* cells have a low initial agglutinability compared with mt− cells and may need more compatible collisions to become stimulated to the level of agglutinability appropriate for pair formation. In a 10% mt* mixture, mt− cells collide less frequently with mt+ cells than in a 10% mt+ mixture. This results in a lower rate of stimulation of mt* cells in a 10% mt− mixture, which is expressed by the slow pair formation in comparison with 10% mt+ mixtures.

The mutual contact-induced stimulation must not be confused with contact-induced gametogenesis that occurs in some unicellular algae (22). The cells that are sensitive to stimulation of agglutinability by flagellar contacts are sexually competent cells that underwent gametogenesis in response to nutrient deficiency (19). Besides, it has been established that there is no mutual influence of the mating types on vegetative development or gametogenesis in Chlamydomonas (19).

An intriguing question is whether the stimulation is due to synthesis of agglutination factor, to transport of agglutination
factor from an intracellular source to the flagella (15) or to redistribution of the constitutive agglutination sites within the flagellar membrane. For C. reinhardtii it has been suggested that concentration of the dispersed agglutination molecules into patches or onto the flagellar tips may lead to better adhesion of the partners (2, 3). Studies with monoclonal antibodies, directed against the agglutination factor, are in progress to test this hypothesis.

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LITERATURE CITED