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The mating-type substances of *Paramecium bursaria*

By L. W. COHEN* and R. W. SIEGEL†

Department of Zoology, University of California, Los Angeles 24, California, U.S.A.

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1. INTRODUCTION

Conjugation occurs in *Paramecium bursaria* when cells of complementary mating types are brought together under appropriate conditions. A system of four mating types is known for syngen 1; cells of each type can conjugate with cells of the remaining three types and normally each pair consists of complementary cells (Larison & Siegel, 1961). Two observations establish the fact that mating is brought about by specific mating-type substances borne on the cilia. First, Jennings (1939) and Metz (1954) showed that cell unions which lead to conjugation are initiated by, and dependent upon, ciliary contacts. Second, if certain cells of complementary mating types are allowed brief ciliary contacts, they may be conditioned so that they will thereafter unite in conjugation with cells of their own mating type (Larison & Siegel, 1961).

Mating-type inheritance and determination are controlled by pairs of alleles at two independently assorting loci in the following way: mating-type I is determined by dominant alleles at loci *A* and *B*; type III is brought about by the double recessive genotype (*aa*/*bb*); types II and IV are respectively genotypes *aa*/*BB* or *aa*/*Bb* and *AA*/*bb* or *Aa*/*bb* (Siegel & Larison, 1960). It seems entirely reasonable that these two loci control in some way the formation of two pairs of complementary mating-type substances carried on the ciliary surfaces. More precisely, the specific combinations of substances carried by the four mating types can be represented **A B**, **a B**, **a b**, and **A b**, respectively so that the complementarity of either **A** and **a** or of **B** and **b** or both can lead to cell attachment and conjugation. Indeed, before a satisfactory genetic analysis of these mating types had been carried out, other considerations had led Metz (1954) to postulate two such pairs of mating-type substances for *P. bursaria*.

The observations to be reported here provide conclusive evidence for the existence of just such pairs of mating-type substances in *P. bursaria*. In these investigations use is made of cell fractions rather than the entire cells, thus opening the way for biochemical investigations on the organelle level.

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2. MATERIAL AND METHODS

The stocks used are listed in Table 1. All are the sexual progeny of four clones originally isolated from a pond in Malibu, California, and have been described elsewhere (Siegel, 1958; Siegel & Larison, 1960; Siegel, 1962).

Methods for handling and culturing *Paramecium bursaria* have been described (Jennings, 1939; Ehret, 1953; Siegel, 1960) and follow closely those used for *P. aurelia* (Sonneborn, 1950).

Cilia were prepared in the following way. Cultures of *Paramecium bursaria* were concentrated to approximately 30,000 animals per ml. by low-speed centrifugation in an International clinical centrifuge. The cells were then disrupted by repeated ejection through a medical syringe fitted with a 25-gauge needle (see Sonneborn, 1950). Counts made with a Petroff-Hauser bacterial counting chamber indicated that approximately 1000 intact cilia were liberated from each cell. Cilia were readily discriminated from other particulates in the brei with the aid of a Zeiss phase-contrast microscope. No intact cells could be found in the brei.

Table 1 *The stocks used in the experiments reported*

Stock	Mating type	Genotype
8	I	*AaBb*
23	II	*aaBB*
51	III	*aabb*
73	I	*AABB*
98	IV	*AAbb*

In order to quantify the observed differences in the intensity of the reaction whereby detached cilia agglutinate to the cilia on other animals, about 150 sexually reactive intact tester cells were placed on a slide in about 0·0025 ml. culture fluid to which 0·06 to 0·1 ml. of brei was added and the preparation was covered with a No. 1 cover slip. Fifteen to twenty-five of the intact animals were observed with phase-contrast (320 ×) in order to detect the agglutination of the detached cilia in the brei with those of the test cells. Numerous trials showed that when no reaction was observed among twenty-five animals, observations of additional animals did not alter the results. For this reason the test was recorded as negative ('−') if twenty-five animals failed to unite with free cilia. Maximum reactions were those in which the entire oral surface, from the anterior to the posterior end, was coated with agglutinating cilia; in weaker reactions the agglutinating cilia coated many but not all of the cilia on the ventro-lateral surface and in other instances detached cilia clung to but one to three of the cilia. These three grades of positive reactions were recorded as '+ + +', '+ +', and '+' respectively.

The thermostability of the agglutinating capacity of detached cilia was tested in the following way: breis were divided into six 0·2 ml. aliquots; the first of these was mixed with the sexually reactive tester cells of complementary mating types to be used in the experiment and only in those cases where '+ + +' reactions were observed was the brei studied further. The second aliquot was allowed to remain

at room temperature during the course of the experiment (90 minutes) and then tested with another sample of the tester cells used in the experiment. The strengths of the reactions for the first and second tests were essentially alike, hence neither the detached untreated cilia nor the intact tester cells lost the ability to react during the course of the experiment. The other four aliquots were transferred to thin-walled glass test tubes and incubated simultaneously in a water bath. After periods of 30, 50, 70 and 90 minutes respectively, one tube was withdrawn, quickly cooled to room temperature and tested.

Where it was desirable to work with the trichocyst-cilia fraction, the cells to be used as the source of cilia were concentrated by low-speed centrifugation and then resuspended twice in 10 ml. samples of distilled water thereby inducing expulsion of many of the trichocysts. Thereafter, the re-concentrated cells were disrupted with a 25-gauge needle and medical syringe, centrifuged in 3 mm. centrifuge tubes for two 4-minute intervals at low speed to remove the endosymbiotic algae and larger cell fragments, and then twice at top speed for 10 minutes to wash and bring down the desired fraction. Resuspensions were in Miyake balanced physiological salt solution (Miyake, 1958) or 'exhausted culture fluid' (Sonneborn, 1950).

3. RESULTS

(i) *The agglutination of detached cilia with animals of complementary mating types*

Only the trichocyst-cilia fraction, separated from other organelles and particulates in a brei by differential centrifugation and repeated washing in Miyake balanced physiological salt solution or 'exhausted culture fluid', showed the capacity to adhere to the cilia present on intact tester cells. Careful observations revealed that the cilia, but not the trichocysts, were involved in these agglutinations. The ability of free cilia to agglutinate to other cells was not decreased by washing the cilia and the tester cells in balanced physiological salt solutions; hence there is no evidence that breis contain a soluble cofactor for the agglutination of the free cilia to the intact tester cells.

A series of observations establishes that the sticking of detached cilia to intact cells is dependent upon the presence of mating-type specific substances on both the detached cilia and the cilia of the intact tester cells. First, positive tests always resulted when the cilia from sexually mature and competent cells were mixed with sexually mature and competent intact cells of any complementary mating type. Second, cilia removed from sexually immature cells never adhered to mature tester cells of complementary types; the converse also resulted in negative tests. Third, cilia removed from sexually mature but sexually incompetent cells—incompetent because of either nutritional conditions or the phase of the daily rhythm of sexuality—never adhered to mature competent testers; the converse also resulted in negative tests. Fourth, if the detached cilia and the tester cells were of the same mating-type agglutinations never occurred. In sum then, positive reactions occur only when conditions necessary for the normal cell unions which lead to conjugation are met. Since such unions define mating-type specificity, the conclusion that if

mating-type specific substances do exist they are associated with the cilia becomes inescapable.

There was no detectable loss in activity of detached cilia maintained at room temperatures for 7 hours. It seems worth while to note that at 4° C. there was no loss in activity in 24 hours, but some loss by 50 hours, and that cilia quickly frozen in an ethanol dry-ice bath and stored at −40° C., retained their activity for two weeks.

(ii) *The effect of trypsin on the agglutination of detached cilia*

In order to further characterize the material basis for the agglutination of detached cilia with cilia on intact tester animals, cilia preparations from cells of 98-IV were treated with trypsin for 36 minutes, washed free of trypsin, and then mixed with tester cells of mating-type II. Salt-free trypsin was dissolved in 0·001 N HCl to give a 1% trypsin solution; this was mixed with 0·06 M borate buffer solution (pH 8·0) and then with the cilia preparation to give a final trypsin concentration of 0·25%. The data shown in Table 2 indicate that trypsin treatment abolishes the ability of the cilia to agglutinate. Microscopic observations of the enzyme-treated cilia revealed that they remained intact and morphologically identical to control cilia.

Table 2. *The agglutination of detached cilia following trypsin treatment. Detached cilia from animals of stock 98-IV; intact tester cells of stock 23-II*

Treatment	Reaction
None	+ + +
Borate buffer	+ + +
0·25% albumin in borate buffer	+ + +
0·25% trypsin in borate buffer	−

(iii) *Effect of heat treatment on the agglutination of detached cilia*

If each mating-type specificity reflects a particular combination of two ciliary substances as postulated by Metz, then some cell unions depend upon one pair of interacting substances, others depend upon a second pair of substances, and still others involve both pairs of substances (see Table 3). The α reaction refers to interaction between the **A** and **a** substances; the β reaction to interaction between the **B** and **b** substances. The evidence that the mating-type substances are proteins together with the fact that related proteins may be distinguished on the basis of their rates of heat denaturation led us to investigate the thermostability of the agents determining ciliary agglutinations as a means of distinguishing the number of substances involved.

The detached cilia from sexually mature competent cells of genotypes *AABB*, *aabb* and *AAbb* were heat treated and mixed with tester cells and the degree of the reaction was scored. The results shown in Table 4 indicate that in each experiment

the postulated alpha reactions are more thermolabile than the beta reactions and that the inactivation of the interactions involving both α and β reactions parallels the inactivation of the more thermostable of the postulated substances.

Table 3. *The basis for the specificity of mating type reactions (after Metz)*

Mating type		I	II	III	IV
	Substances present	**A B**	**a B**	**a b**	**A b**
I	**A B**	—	α	α, β	β
II	**a B**		—	β	α, β
III	**a b**			—	α
IV	**A b**				—

As an additional test, it was postulated that the agglutination of detached cilia from cells of genotypes *AAbb* and *aaBB* with tester cells of genotype *aabb* depends upon an alpha reaction in one case and a beta reaction in the other. Experimentally the alpha reaction was found to be more thermolabile than the beta (see Table 5) in agreement with our first results.

Table 4. *Ciliary agglutination following heat-treatment of detached cilia*

Experiment number	Genotype of cells: Detached cilia	Genotype of cells: Intact testers	Reaction	Degree of reaction after min. heat: 0	30	50	70	90
1	*AABB*	*aabb*	α, β	+ + +	+ + +	+ + +	+ +	−
		aaBB	α	+ + +	+	−	−	−
		AAbb	β	+ + +	+ + +	+ + +	+	−
2	*aabb*	*AABB*	α, β	+ + +	+ + +	+ + +	+ + +	+ +
		AAbb	α	+ + +	+ + +	+ +	+	−
		aaBB	β	+ + +	+ + +	+ + +	+ + +	+ +
3	*AAbb*	*aaBB*	α, β	+ + +	+ + +	+ + +	+ + +	+
		aabb	α	+ + +	+	−	−	−
		AaBb	β	+ + +	+ + +	+ + +	+ + +	+

(1) 45·5° C., (2) 44·5° C., (3) 44·8° C.

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Table 5. *A comparison of the thermostability of the postulated* **A** *and* **B** *mating-type substances at 45·2° C*

Genotype of cells			Degree of reaction after min. heat				
Detached cilia	Intact testers	Reaction	0	30	50	70	90
AAbb	*aabb*	α	+ + +	+	−	−	−
aaBB	*aabb*	β	+ + +	+ + +	+ + +	+ + +	+ + +

(iv) *Region of cell surface involved in ciliary agglutinations*

The agglutination of free cilia to intact animals is a sensitive test for the area on the animal which carries the mating-type substances, for the attachment of but one cilium can be detected. The most frequent site for attachment is the ventro-lateral surface, that is, the surface which is differentiated into the oral groove. If the reaction is particularly strong, cilia may also be found attached to the anterior-most cilia on the adoral surface. Depending on several factors, cells may vary in their readiness to mate; some form lasting unions upon initial contact while others join so loosely that the swimming movements will uncouple them. Observations on the union of free cilia with intact cells suggest that the area of reactive cilia is increased anteriorly from its posterior ventral origin as the intensity of mating readiness increases. The specialized cilia in the gullet region do not appear to carry mating-type substances. These observations confirm those made by Hiwatashi (1961) for *P. caudatum*, namely, that the ventral halves of bisected animals generally have the ability to 'mate' and that in the infrequent cases where dorsal halves react, unions are limited to the anterior dorsal tip. However, in contrast to Hiwatashi's observations for *P. caudatum*, where the most frequent region of contact in weak reactions of whole animals is the anterior ventral surface, weak reactions in *P. bursaria* are limited to the posterior ventral-lateral region.

Free cilia were not found sticking to the lateral dorsal surface; presumably the cilia there do not carry the mating-type substances. Much less than half and perhaps only one-tenth of all the cilia on a cell may carry mating-type substances. Breis of two complementary mating types were mixed in an effort to observe direct interaction between the excised cilia. Our inability to recognize ciliary agglutinations in these mixtures may be due to the small number of cilia carrying the mating-type substances or to presently unknown additional factors.

4. DISCUSSION

Jennings (1939) observed that the initial phase of the mating reaction is the adhesion of cells due to ciliary unions. Sonneborn (1942) and Metz (1954) found that cells which had been temporarily united with individuals of a complementary mating type behaved as if they had acquired the complementary mating-type specificity in the course of contact for they subsequently united with cells of their

own original mating type. These facts led to the suggestion that special mating-type substances, borne on the cilia, are responsible for specific cell unions which lead to conjugation. Metz and his co-workers studied the loss of mating capacity following a variety of chemical treatments and found some evidence that the substances were proteins. Knowledge of the genetics of mating types and the basis for cell unions in *P. bursaria* (Siegel & Larison, 1960; Larison & Siegel, 1961) together with the provacative hypothesis of Metz led to the investigations which have been reported here.

Taken together, the data strongly suggest that dominant and recessive alleles at the *A*-locus directly or indirectly control a pair of complementary substances **A** and **a**; alleles at the *B*-locus control a second pair of substances **B** and **b**. Admittedly the data do not permit differentiation between the members of each pair. Each of the four mating types is characterized by a unique combination of two

Table 6. *Mating types, genotypes and mating-type substances*

		Mating type substances present	
Mating type	Genotype	Alpha	Beta
I	*AABB* *AaBB* *AABb* *AaBb*	**A**	**B**
II	*aaBB* *aaBb*	**a**	**B**
III	*aabb*	**a**	**b**
IV	*AAbb* *Aabb*	**A**	**b**

substances as shown in Table 6. Cell unions can be brought about by the interaction of a single pair of complementary substances (**A** and **a** or **B** and **b**) or by the interaction of both pairs of substances as shown in Table 3.

We have used the word 'complementary' in referring to the mating-type substances, implying that the interaction between a pair of substances involves simply a steric fitting (or antigen-antibody type of union) rather than an enzymatically mediated interaction. There are two reasons for suggesting this interpretation. First, adhesion between two cells of complementary mating types appears to be instantaneous. Second, immediate ciliary union is temperature independent in the range 4° C to 27° C.

The striking fact that the cilia on the oral surface of *P. bursaria* possess mating-type substances while those on the adoral surface do not invites new investigations of patterns of intracellular differentiation. The question whether a given cilium carries one or two substances remains unanswered.

Breis prepared from cells which would not mate—by reason of their life-cycle stage (sexual immaturity) or the period of the daily rhythm of reactivity—failed to react in any way with appropriate tester cells. Apparently then the inability to mate reflects the complete absence of active mating-type substances. Possibly, during the period of sexual immaturity the substances are not produced; then later in the life-cycle, during the period of maturity, the substances are formed and disappear in a regular daily cycle.

5. SUMMARY

The detached cilia from sexually reactive cells of *Paramecium bursaria* will agglutinate with the cilia of intact sexually reactive animals of a complementary mating type. No such reaction will occur if incompetent cells are used or if the cells are of the same mating type. Particulates other than cilia do not adhere to tester cells; the cilia which carry the specific mating-type substances are restricted to the ventro-lateral surface of the animal.

Studies of the heat inactivation of the ability of detached cilia to agglutinate confirm in detail the hypothesis of Metz which holds that cell unions leading to conjugation are brought about by the interaction of two pairs of complementary substances, **A** and **a** and **B** and **b**; the former pair of substances is more heat labile than the latter. The data suggest that animals of each of the four mating types carry a unique combination of two substances, namely, **AB**, **aB**, **ab**, and **Ab**.

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