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Gamones and mating types in the genus *Blepharisma* and their possible taxonomic application

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SUMMARY

Mating types I and II of *Blepharisma japonicum v. intermedium* excrete gamones 1 (blepharmone J) and 2 (blepharismone) respectively. The gamone of one type transforms cells of the other type so that they can conjugate with each other. We found that three other species, *B. americanum*, *B. musculus* and *B. stoltei*, have two types of cells homologous to those in *B. japonicum*; one (type II) excretes a factor which has the same activity as gamone 2 of *B. japonicum*, the other (type I) responds to this gamone by cell union. Type I cells of these species also excrete a gamone which induces pairs in type II cells of particular strains. Complementarity for mating is observed in some combinations of the two types.

These results indicate that each of the four species has at least one pair of complementary mating types, I and II, with the gamones of the type II's being the same molecule, blepharismone, while gamones of type I's are species- or syngen-specific blepharmone. These generic and specific gamones can be utilized to clarify existing taxonomic and evolutionary questions in the genus *Blepharisma*.

1. INTRODUCTION

Mating-type differences in ciliates were first discovered in *Paramecium aurelia* (Sonneborn, 1937). Complementary mating types can mate with each other, assuring the gene flow in a population consisting of these mating types, while the high specificity of the complementarity excludes or restricts the gene flow between such populations or syngens (Sonneborn, 1957). The separation of morphologically similar ciliates into sibling species has recently been analysed by Sonneborn (1975). Further investigations into the mechanisms leading to reproductive isolation can be expected to deepen our understanding of evolutionary processes.

The isolation and identification of the chemical basis of mating-type specificity is one step in this direction. These have been occasionally attempted in *Paramecium* (Metz, 1954; Hiwatashi, 1969), but the complete isolation of such substances among the ciliates has been carried out only in *Blepharisma*.

B. japonicum v. intermedium differentiates into mating types I and II (Miyake & Beyer, 1973). Type I cells excrete gamone 1 which transforms type II cells into competency for pairing. Type II cells excrete gamone 2 which similarly transforms type I cells. Each gamone promotes the production of the other. Gamone 1, blephar-

mone, is a glycoprotein of 20 000 daltons (Miyake & Beyer, 1974; Braun & Miyake, 1975). Gamone 2, blepharismone, is calcium-3-(2′-formylamino-5′-hydroxybenzoyl)-lactate (Kubota *et al.* 1973). This gamone is also an attractant of type I cells (Honda & Miyake, 1975).

Some other species of *Blepharisma* excrete a factor with blepharismone activity or respond to blepharismone by cell union (Miyake, 1968, 1974). This suggests that a mating-type system similar to that of *B. japonicum* might be found in other species of this genus and that they might share blepharismone as one of their gamones. This investigation was carried out to test these hypotheses and to probe into the genetic structure of the genus *Blepharisma*.

2. MATERIALS AND METHODS

The following species and strains were used: *B. americanum*: Berlin, Bloomington (Bloom), McManus (McMan), Monterey (Mont), North Carolina Old (N.C.), N.Y.U., 611 and 865; *B. musculus*: Leningrad (Lenin), Seshachari (SES), Sinuosum (Sin), Ward and 622; *B. japonicum*: Bangalore (Bangal), Bangalore albino and Niigata (Nii); *B. stoltei*: Federsee (Feder) and Nara. Unidentified species: Connecticut Valley (C.V.), Cp3 long (Cp3l), Cp6 short (Cp6s) and Rome I (Rome). Clones A1 of Bangal albino and clone 4a of Bangal were reference mating types I and II respectively.

All the strains except C.V., which was from the Connecticut Valley Biological Supply Company, Southampton, Mass., were supplied by Dr H. I. Hirshfield from his collection at New York University. Bangal and Bangal albino were from Dr F. Inaba, Nara Women's University, Nara.

Classification was mainly based on the recent work by Hirshfield, Isquith & Dilorenzo (1973). The Bangalore strains, on which most of the studies on gamones were carried out, were previously classified as *B. intermedium*, but are now reclassified as *B. japonicum v. intermedium*, a variety of *B. japonicum*.

General techniques for culture and handling were those described by Sonneborn (1970) for *Paramecium*. Single cell clones of each strain were grown at 25 °C into a 1 l culture on a modified lettuce-juice medium inoculated with *Aerobacter aerogenes* (Miyake & Beyer, 1973). Some of the mating type II cultures of Bangal were grown at 30 °C or under oscillating temperatures (28–36 °C, 4–5 cycles/day). These conditions favoured the expression of mating type II in some cultures of *B. japonicum* (Beyer & Miyake, unpublished).

When growth of a culture stopped or nearly stopped, cells were concentrated, washed with and suspended in SMB, a synthetic non-nutrient medium for *Blepharisma* (Miyake & Beyer, 1973), at the cell density of $2–10 \times 10^3$/ml, and kept for 1 day or longer before use. Mixing of cells with gamone, cell-free fluids or with cells of other cultures was carried out in a slide depression which can hold 1 ml of medium. Pair formation was recorded by an arbitrary scale of 0–6, where each unit represents no pairs, approximately ~2, 5, 10, 20, 40 and 80% united cells, respectively.

Selfing, the conjugation within a culture derived from a single cell, often occurred

in control cells which were suspended in SMB. When it occurred in the experiment testing the cell-union-inducing effect of gamone or cell-free fluid, the effect was regarded as positive if pair formation of unit 4 or higher was observed before selfing began in the control, or if pair formation in the treated cells was at least 2 units higher than that in the control any time during the observation.

Gamone 1 was blepharmone partially purified by a Bio-Gel 150 column as indicated (Miyake & Beyer, 1973) and was used as a SMB solution of 10^6 units/ml activity. Gamone 2 was synthetic blepharismone (Tokoroyama, Hori & Kubota, 1973) purified as indicated (Kubota *et al.* 1973) and was used as a SMB solution of $1{\cdot}6 \times 10^4$ units/ml activity. The unit activity is defined as the smallest amount of gamone activity that can induce at least one homotypic cell union in 500 cells suspended in 1 ml SMB (Miyake & Beyer, 1973).

3. RESULTS

(i) *Response of various strains to gamones of* B. japonicum v. intermedium

A cell suspension of the strain to be tested was mixed with gamone solution in a 99:1 ratio and observed for 2 days for the induction of pairs (Table 1). Gamone 1 induced pairs only in mating type II of Bangal, the strain from which this gamone was obtained, indicating a high specificity of this gamone. On the contrary, gamone 2 induced pairs in about two-thirds of the strains covering all the four species tested. This result confirms and extends the previous observation (Miyake, 1974) that species other than *B. japonicum* can respond to gamone 2 by pair formation. In Bloom, Mont, C.V. and Nara, the effect of gamones could not be detected because of massive selfing.

(ii) *Excretion of factors with the activity of gamones of* B. japonicum v. intermedium *by various strains*

Cell suspensions of mating types I and II of Bangal were mixed with the cell-free fluid of all the strains in a 1:1 ratio and observed for 2 days for the induction of pairs (Table 1). Pairs were induced in type I cells by the cell-free fluids of Berlin, N.C., Bloom, Mont, Ward, Cp6s, Cp3l, C.V., Feder, Nara and Bangal. These strains therefore excreted a factor with gamone 2 activity. Pairs were induced in type II cells by cell-free fluids of Feder, Nii and Bangal. These strains therefore excreted a factor with gamone 1 activity.

The factor with gamone 2 activity was thus excreted by all four species tested, confirming and enlarging previous observations (Miyake, 1968, 1974) that species other than *B. japonicum* excrete such a factor. On the other hand, the factor with gamone 1 activity was excreted only by Feder, Nii and Bangal. Nii was once classified as *B. microstomata* but later was reclassified as *B. japonicum v. microstomata* (Hirshfield, Isquith & Dilorenzo 1973). This leaves Feder as the only example of the excretion of the factor with gamone 1 activity by a species other than *B. japonicum*.

Table 1. *Response to gamones 1 and 2 of* Blepharisma japonicum v. intermedium *and the excretion of a factor with the activity of these gamones in various strains* of Blepharisma

(In addition to Bangal mating type I (mt I) and mating type II (mt II), a selfing culture of this strain is included as Bangal. For the response to gamones and the excretion of the factor: +, positive response or excretion; −, no response or no excretion; ?, response not detectable because of massive selfing. For selfing: +, more than 5% paired cells; (+), less than 5% paired cells; −, no paired cells.)

Strain and *Blepharisma* sp.	Response to		Excreted factor with the activity of		Selfing
	Gamone 1	Gamone 2	Gamone 1	Gamone 2	
B. americanum (III)*					
N.Y.U.	−	−	−	−	−
McMan	−	+	−	−	−
611	−	+	−	−	(+)
865	−	+	−	−	(+)
Berlin	−	+	−	+	+
N.C.	−	+	−	+	+
Bloom	?	?	−	+	+
Mont	?	?	−	+	+
B. musculus (III)					
Lenin	−	−	−	−	−
622	−	+	−	−	−
SES	−	+	−	−	(+)
Sin	−	+	−	−	(+)
Ward	−	+	−	+	+
Unidentified (III)					
Rome	−	+	−	−	−
Cp6s	−	−	−	+	+
Cp3l	−	+	−	+	+
C.V.	?	?	−	+	+
B. stoltei (IV)					
Feder	−	+	+	+	+
Nara	?	?	−	+	+
B. japonicum (IV)					
Bangal mt I	−	+	+	−	−
Bangal mt II	+	−	−	−	−
Bangal	?	?	+	+	+
Nii	−	+	+	−	−

* ( ), Megakaryotype shown as III or IV.

(iii) *Interaction between strains*

Strains 611, Berlin, Bloom, Mont, Lenin, SES, Ward, Cp6s, Cp3l, C.V. and Feder were mixed two by two in all possible combinations and observed for 3 days for the induction of pairs. Massive selfing in the unmixed controls except those of Lenin, SES and 611 made it difficult to obtain convincing results in many mixtures. Therefore only the results on these three strains will be described. Lenin did not undergo selfing, while in SES and 611 less than 5% of the cells underwent selfing.

Of the three possible combinations, only Lenin × SES produced pairs. They both belong to *B. musculus* suggesting that they are complementary mating types of this species. As SES responds to gamone 2 (Table 1), this strain may be regarded as type I and therefore Lenin as type II. When Lenin was mixed with 865, N.C., Sin and Rome in which selfing was not massive, mixtures with Sin and 865 produced pairs. Both Sin and 865 responded to gamone 2 (Table 1) and therefore they may be regarded as type I, indicating again that Lenin is type II. Sin is *B. musculus*, but 865 is classified as *B. americanum*.

All the strains, except for N.Y.U., McMan and 622 were mixed with Bangal type I (albino) and type II. Pair induction was looked for during 2 days' observation. Homotypic pairs of Bangals could be distinguished either by colour (I–I) or size + density of colouration (II–II). Similarly, disparate pairs indicated interstrain pairing.

Homotypic pairs of type I were induced by Berlin, N.C., Bloom, Mont, Ward, Cp3l, C.V., Feder and Nara. Because all these strains excreted a factor with gamone 2 activity (Table 1), the type I pairs were induced by this factor. Interstrain pairing occurred between Bangal type I and Berlin, Bloom, Mont, Ward, Cp3l, C.V., Feder and Nara. Because all these strains excreted a factor with gamone 2 activity and also underwent selfing (Table 1), it may be concluded that the interstrain pairs were induced by gamone 2 activity which transformed type I cells. The transformed type I cells could then unite with cells of the other strains whose competence for union is indicated by the occurrence of selfing.

Homotypic pairs of type II were induced by Berlin, Feder and Nii. Because Feder and Nii excreted a factor with gamone 1 activity (Table 1), the type II pairs induced by these strains were the response to this factor. Interstrain pairing occurred between Bangal type II and Berlin, C.V., Feder and Nii. Because Feder and Nii excreted a factor with gamone 1 activity and responded to gamone 2 by cell union (Table 1), it may be concluced that the interstrain pairs with these strains were induced by gamone 1 activity which transformed Bangal type II cells into competency for pairing and also activated them to excrete gamone 2. This gamone in turn transformed Feder and Nii, resulting in interstrain pairing. Cell unions of Bangal type II in the mixtures with Berlin and C.V. need further investigation because only a small number of Bangal cells united weakly and temporarily.

(iv) *Excretion of gamones other than gamones 1 and 2 of* B. japonicum v. intermedium

Strains 611, Berlin, Bloom, Mont, SES, Ward, Cp3l and C.V. were incubated with gamone 2 (160 units/ml) and 0·01 % bovine serum albumin for 1 day. Gamone 2 and albumin, the inducer and stabilizer of gamone 1 respectively (Miyake & Beyer, 1973), were used for their possible favourable effect in detecting new gamones. Their cell-free fluids were then mixed with cell suspensions of 611, 865, N.C., Lenin, Sin, Rome and Cp6s in a 1:1 ratio and the induction of pairs was observed for 2 days (Table 2).

Two distinctive classes were observed. The first was pairing induced in Lenin by cell-free fluid of SES. The second was the response of Cp6s to cell-free fluids of

Berlin, Bloom, Mont, Ward and Cp3l. As Lenin and Cp6s responded to neither gamone 1 nor 2 of *B. japonicum* (Table 1), these inductions must be due to two new and different gamones. Since SES and Lenin were assumed to be types I and II of *B. musculus* respectively in the previous section, the gamone which was excreted by SES and induced pairs in Lenin may be regarded as gamone 1 of *B. musculus*. Similarly, the gamone which induced pairs in Cp6s may be regarded as gamone 1 of *B. americanum*, to which belong Berlin, Bloom and Mont. This result suggests that the heretofore unidentified strains Cp3l and Cp6s are also *B. americanum*. Although SES, Ward and Lenin are all *B. musculus*, the cell-free fluid of Ward induced pairs in Cp6s but not in Lenin, while SES fluid had the reverse effect. This suggests that *B. musculus* has at least two syngens, 1 and 2, to which SES and Ward belong, respectively, and that gamone 1 of syngen 2 is syngen-specific although it cross-reacts to *B. americanum*.

Table 2. *Induction of pairs by cell-free fluids in strains of megakaryotype III of* Blepharisma

(+, Positive induction; −, no induction; blank, not tested.)

Cell-free fluid	Cells						
	611	865	N.C.	Lenin	Sin	Rome	Cp6s
611	−			−			−
Berlin	−			−			+
Bloom		−	−	−	−	−	+
Mont		−	−	−	−	−	+
SES	−	−	−	+	−	−	−
Ward	−	−	−	−	−	−	+
Cp3l	−			−			+
C.V.		−	−	−	−	−	

4. DISCUSSION

Mating type I of *B. japonicum* is characterized by the excretion of gamone 1 and by the ability to respond to gamone 2 by cell union. Mating type II is characterized by the excretion of gamone 2 and by the ability to respond to gamone 1 by cell union. Therefore, if a strain excretes a factor with the activity of gamone 1 and/or responds to gamone 2 by cell union, it may be regarded as the mating type which is the same or homologous to mating type I of *B. japonicum*. Similarly, if a strain excretes a factor with the activity of gamone 2 and/or responds to gamone 1 by cell union, it may be regarded as the mating type which is the same or homologous to mating type II of *B. japonicum*. Factors excreted by types I and II thus classified may be regarded as gamones which are the same or homologous to gamones 1 and 2 of *B. japonicum* respectively.

Most of the strains tested were classified in this way as type I (McMan, 611, 865, 622, SES, Sin, Rome), type II (Cp6s) or a mixture of the two types (Berlin, N.C., Ward, Cp3l, Feder). Lenin was classified as type II because of its complementarity to 865, SES and Sin. In Bloom, Mont, C.V. and Nara, the presence of type II cells

was detected by the excretion of a factor with gamone 2 activity. In these strains, type I cells which respond to gamone 2 could not be detected because of massive selfing. However, Bloom and Mont excreted a gamone which induced pairs in Cp6s (type II) and therefore may be regarded to also contain type I cells.

The detection of type I cells was all based on the response to gamone 2 of *B. japonicum* with the exceptions of Bloom and Mont. The detection of type II cells was all based on the excretion of a factor with the activity of gamone 2. The extensive cross-reaction of gamone 2 and the ubiquitous excretion of a factor with the activity of gamone 2 strongly suggest that all these factors with gamone 2 activity are the same molecule, blepharismone. This is still to be chemically confirmed, but the relative simplicity of the blepharismone molecule, a derivative of tryptophan, supports this assumption.

In contrast, gamone 1 of *B. japonicum* did not cross-react at all. Of all the strains of the other three species, only Feder excreted a factor with gamone 1 activity. However, the excretion of gamones homologous to gamone 1 of *B. japonicum* by other strains was demonstrated by (1) the cell-free fluid of SES (type I) which specifically induced pairs in Lenin (type II), and (2) the cell-free fluids of Berlin, Bloom, Mont, Ward and Cp3l, all of which specifically induced pairs in Cp6s (type II). In this connexion it may be noted that all the strains which excreted the factor with gamone 2 activity were selfers (Table 1), presupposing the presence of both types of cells. This suggests that gamone 2 was induced by a gamone of type I as in *B. japonicum*. These gamones of type I cells may be called blepharmones A, J, M and S as they are excreted by *B. americanum*, *B. japonicum*, *B. musculus* and *B. stoltei* respectively.

The following aspect of the genetic structure of the genus *Blepharisma* emerges. Each species consists of at least a pair of complementary mating types, I and II. In many species the gamone of type II is the same molecule, blepharismone, while each type I has species- or syngen-specific blepharmone.

How many of about 20 known species of *Blepharisma* use blepharismone as gamone 2 is to be investigated in the future. If other molecules are also used by some species, large taxonomic groupings may be made on the basis of the gamone 2 molecule. The more detailed structure of the genus may be obtained by the comparative investigation of blepharmone, the specificity of which is much higher. For example, the cross-reactions of blepharmone S to *B. japonicum* and of blepharmone A to *B. musculus* indicate closer relations between *B. japonicum* and *B. stoltei* on one hand and between *B. americanum* and *B. musculus* on the other. This supports the new classification proposed by Hirshfield, Isquith & Dilorenzo (1973), in which the former two and the latter two species are placed in different megakaryotypes, IV and III, respectively.

The striking chemical difference between gamones 1 and 2 deserves special consideration. If the two pairs of gamone-receptor systems of *Blepharisma* developed at different times during the evolution of this genus, the gamone 2 (G2)–receptor (R2) system probably developed first, because this system is present in all four species tested. At that time G2 was the mating signal common for all cells. When the

gamone 1 (G1)–receptor (R1) system evolved later, each cell then had G1, G2, R1 and R2, and it could eliminate or suppress one of the receptors and/or one of the gamones without losing the capacity to interact for mating. Two types of cells having G1–R2 and G2–R1, respectively, would have been thus formed to produce complementary mating types. If more gamone-receptor systems accumulate, a multiple mating type system may develop.

Sexual isolation would result from mutations of gamones and receptors. The modification of blepharmone can be achieved by simply substituting amino acids, while changes in a simple molecule like blepharismone require new enzymes. This can explain the present condition of *Blepharisma* in which only one of a pair of gamones, blepharmone, is species-specific. As they still share blepharismone as gamone 2, their sexual isolation is incomplete. Interspecific crosses may occur as suggested by the pair formation between *B. japonicum* and other species (Result iii). Conjugation between Nii (*B. japonicum*) and Nara (*B. stoltei*) produced some progenies (Inaba, 1965). The close relationship between all strains of *Blepharisma* which makes the classification of this genus difficult, the point stressed by Hirshfield, Isquith & Dilorenzo (1973), might be due to this situation.

A question may be raised whether there exist ciliates which still possess only one gamone-receptor system. In such ciliates conjugation may occur but mating types would never be found because each cell uses its own gamone as the mating signal for itself. This kind of selfing may also occur if the separation of gamone and its receptor is incomplete in ciliates possessing more than two gamone-receptor systems. In some ciliates including *B. japonicum* each cell has a genetic potentiality to express more than one mating type and the differentiation of mating type is achieved by suppressing some of the potentialities (Sonneborn, 1947; Nanney, Caughey & Tefankjian, 1955; Bleyman, 1967). If this control mechanism is not perfect, cells may respond to their own gamone. In these cases the knowledge of the chemical basis of the mating signal will be indispensable to reveal incompletely differentiated mating types.

Gamones have not been isolated in other ciliates, though evidence indicates that gamones of *Paramecium* are cell-bound proteins (Metz, 1954; Hiwatashi, 1969). Excreted gamones or gamone-like substances have been reported only sporadically in *Euplotes patella* (Kimball, 1939), *Tetrahymena pyriformis* (Phillips, 1971), *Tokophrya* (T. M. Sonneborn, personal communication cited by Miyake, 1974) and *Oxytricha bifaria* (Ricci, Esposito & Nobili, 1975). Their chemical nature is unknown. More excreted gamones might be found in the about 70 known genera of heterotrichous ciliates to which *Blepharisma* belongs but in which mating type has been very poorly investigated. A search for excreted gamones, especially chemically simple ones, might provide a powerful tool in establishing phylogenic relations in ciliates.

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