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Biology of *Euplotes focardii*, an Antarctic ciliate

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Abstract. *Euplotes focardii*, a ciliate species recently collected from sand sediments of Terra Nova Bay (Ross Sea, Antarctica) reproduced in the laboratory with a duplication time of approximately 72 h, at 4°C. Strains representative of two different mating types were identified and mixed together to produce mating pairs. These showed traits rather unusual for *Euplotes* species. The two pair members remained united for at least 8–10 days. However, only one carried out fertilization and was able to give rise to a new clone of vegetative cells; the other underwent cell body shrinking after 4–5 days of union, lost the locomotory ciliary apparatus, and eventually died. By analyses of mating pairs formed in mixtures of cell samples cytologically distinct from each other, it was ascertained that the different cell behavior is strain-specific.

In the study of the biology of Antarctic organisms, Protozoa have so far been largely neglected. Yet, from an ecological point of view, they represent one of the most important components of the sea ice and benthonic communities, for both the variety of species and number of individuals (Buck and Garrison 1983; Corliss and Snyder 1986; Dillon et al. 1968; Thompson 1972; Thompson and Croom 1978). In addition, they may provide unique systems to investigate cell properties and structures underlying elementary strategies of life and adaptive mechanisms (Nanney 1980). Indeed, they represent individual cells directly exposed to natural selection.

Some interstitial hypotrich ciliates have been collected from Terra Nova Bay of Ross Sea (Valbonesi and Luporini 1990a–c) and strains representative of *Euplotes focardii*, described as a new species (Valbonesi and Luporini 1990a), in addition to reproducing well in captivity, proved to be genetically different and capable of regulating the onset of the sexual process of mating (or conjugation). Therefore, these strains represent suitable material for approaching a study of the biology of an Antarctic species of ciliate under controlled laboratory conditions; here we describe basic aspects of the vegetative reproduction and mating.

Materials and methods

Sample collection and cell culture

The samples of sea water and sandy sediment from which *Euplotes focardii* Valbonesi and Luporini, 1990, was isolated were collected from a small cove near the Italian Antarctic base located at Terra Nova Bay (Ross Sea, 74°42′S, 164°06′ E), by means of a "Petersen" dredge hauled at a depth of 7 m, at the beginning of 1988 and 1989. At the time of collection, the following environmental parameters were recorded: salinity, 35‰; temperature, −1.8°C; pH, 8.1–8.2. All the original wild-type strains and offspring clones were grown in sterilized natural sea water and usually fed with the green alga *Dunaliella tertiolecta* or unidentified bacteria (present in the same Antarctic samples and allowed to multiply following suspension with a Luria-Bertani medium composed of 1% Bacto Tryptone and 0.5% yeast extract). They were maintained in a cold room, at 4°C, with a rhythm of 16 h of darkness and 8 h of very weak light. One strain denominated TN_1 has been deposited, as a living reference strain, at the Culture Collection of Algae and Protozoa (The Ferry House, Ambleside, Cumbria LA22 OLP, UK), where it is accessible under the number CCAP 1624/17.

Optical and scanning electron microscopy

For analyses of the vegetative and mating stages of the cell nuclear apparatus, cells were fixed with a mixture of one part of ethanol and three parts of acetic acid, air-dried, stained with 4′6-diamidino-2-phenylindole 2HCl (DAPI) at a concentration of 1 μg/ml, and observed with a reflected light fluorescence microscope (Olympus, exciter filter UG1). Cells for scanning electron microscopy observations were fixed for 30 min, at 4°C, using a modified Parducz solution made by mixing six parts of 2% OsO_4 (w/v) in sea water and one part of saturated aqueous solution of $HgCl_2$. Fixed cells were then processed by standard procedures, as previously described in detail (Valbonesi and Luporini 1990b), and observed with a Stereoscan 200 (Cambridge Instruments Ltd).

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Mating mixtures

For the induction of mating pairs, moderately starved cell samples were usually mixed at concentrations of about 200 cells/ml in three-depression spot slides.

Autoradiography

For radiolabeling of the cell nuclear apparatus, [^{3}H]-thymidine (the radioactive Centre, Amersham), at a specific activity of 23 Ci/mmole diluted with sea water to 10 μCi/ml, was used. After an incubation of 3 days, cells were prepared for autoradiographic analysis with standard procedures, as described by Luporini and Bracchi (1973).

Results

Reproduction

Healthy reproducing cells of standard dimensions (ca. 70–80 μm in length and 50–60 μm in width) were observed only in cultures maintained at a concentration of 100–200 cells/ml and exposed to a temperature ranging from -2°C to $+8$°C. Temperature values over 10–11°C caused cells to become irreversibly damaged. This damage was first evident by abnormal cell body swelling and by a drop in cell motility. Cells never divided and finally died. Formation of resting stages, such as cysts, was never observed.

At 4°C, the temperature eventually chosen for *E. focardii* reproduction under laboratory conditions, the cell duplication time was 68–76 h, with a mean of 72 h 35 min. This determination was carried out on several samples of 100 dividing cells which were isolated from growing cultures, suspended individually in the presence of food, and monitored for the appearance of the next fission furrow. The duration of cell cycle stages was similarly studied, based on cytological analyses of the nuclear apparatus of aliquots of cells which were removed seriatim from cultures of putatively synchronous cells (again obtained starting from groups of dividing cells). The G_1, S, and G_2-D stages were identified on the basis of the shape and presence or absence of the DNA replication bands of the cell macronucleus, as described by Gall (1959), and found to average 60 h (equivalent to 83.3% of the cell duplication time), 9 h (12.5%), and 3 h (4.2%), respectively.

Mating

Mating pairs were never observed to form "spontaneously" in sib-cell cultures (a phenomenon usually described as "selfing"). They appeared only in cell mixtures prepared by combining strains of the 1988 collection with those of the 1989 collection. Mixtures between strains of the same collection did not produce pairs, thus implying identical genetic origins from division products of a single

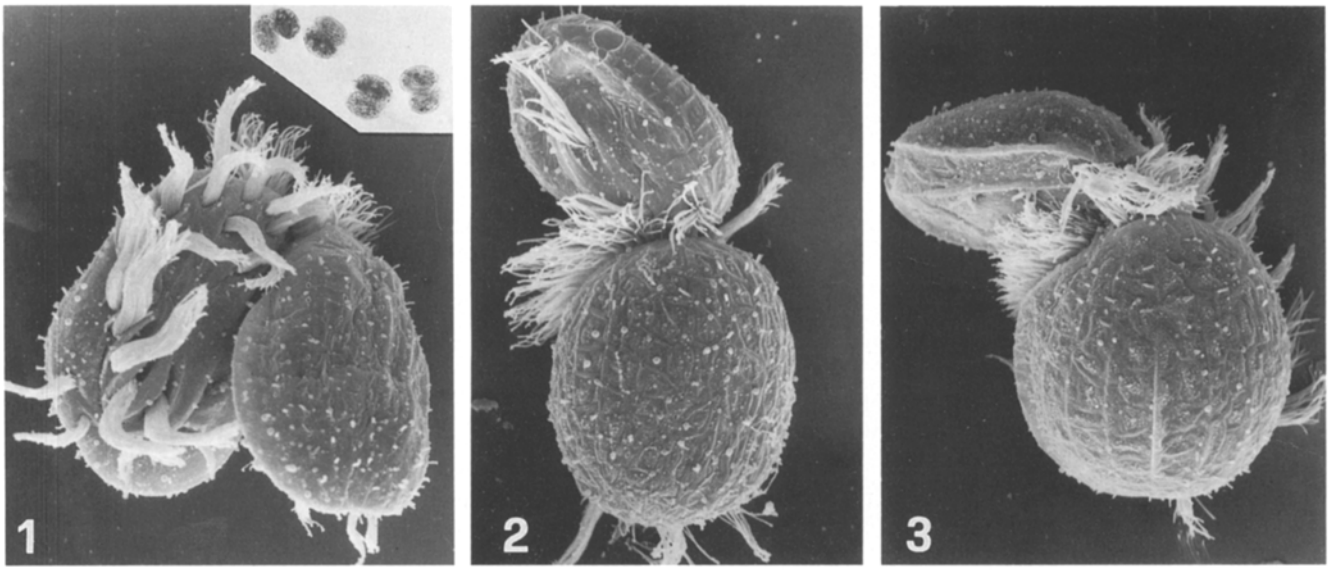

Fig. 1. Scanning electron micrograph of a 4-day old mating pair of *E. focardii*. The defective member (on the viewer's right) already appears with reduced cell body dimensions with respect to the normal partner. ×550. *Inset*: initial mating pairs as seen, *in vivo*, with an optical microscope. ×70. (The differences in the "transparency" of the cytoplasm between members of the same pair are due to different feeding regimens).

Fig. 2. Scanning electron micrograph of a 6-day old mating pair of *E. focardii*. The defective member shows, in addition to a marked shrinking of the cell body, the loss of most ciliary organelles (cirri) on the ventral surface. ×550

Fig. 3. Scanning electron micrograph of a 8-day old mating pair of *E. focardii*. The defective member shows the ventral surface devoid of cirri and is loosely attached, as a dead cell body, to the normal partner. ×550

isolate from the wild (samples being monitored in the laboratory about three months after their collection). Eventually, strains TN_4 (of the 1988 collection) and TN_{15} (of the 1989 collection) were chosen to represent two different mating types, denominated Mt-I and MT-II respectively, and used in all the following experiments.

Mating pairs of *E. focardii*, the great majority of which proved to be heterotypic between Mt-I and Mt-II cells (as described below), appeared to be very unusual in comparison with all the other *Euplotes* species studied so far. The stages of cell mating union and post-mating reorganization were extremely long-lasting, at least 8–10 days and 13–15 days, respectively. (The post-mating reorganization stage was taken as the time between appearance of the primordium of the new macronucleus in one of the two mating partners and the first cell division marking the beginning of the new clonal life cycle). Mating pairs became markedly "asymmetric" after 4–5 days of union, due to dramatic alterations in the morphology and nuclear behavior of one of the two mating partners.

At a morphological level, as illustrated in Figs. 1–3, the most apparent alterations involved a progressive decrease in cell volume (considered as a half ellipsoid) of one partner to about one fifth of the original and a concomitant loss (or degeneration?) of most cirri and adoral membranelles. Each mating pair, from over hundreds examined, eventually consisted of one apparently normal mate and one loosely attached, small, and inactive partner which was destined to die soon after separation. At the nuclear level, as schematically illustrated in Fig. 4, the preliminary mitosis and two subsequent meiotic divisions, usual in *Euplotes* species, were observed to be carried out by both partners of the pair. Following this stage, we were unable, mainly due to a precocious and extensive fragmentation of the macronucleus, to define how many of the eight gonal products of the normal mate partner were destroyed and which one divides again to produce the two (migratory and stationary) gamete nuclei. In the defective partner, corresponding with the beginning of the morphological alterations, the micronuclear development was first delayed and then arrested; only occasionally, the final stage of two gamete pronuclei was observed. The primordium (or "anlagen") of the new macronucleus developed only in the ex-conjugant derived from the normal mating partner, and it was presumed that its origin was autogamic (i.e., derived from the fusion of the two gamete nuclei produced by the same cell).

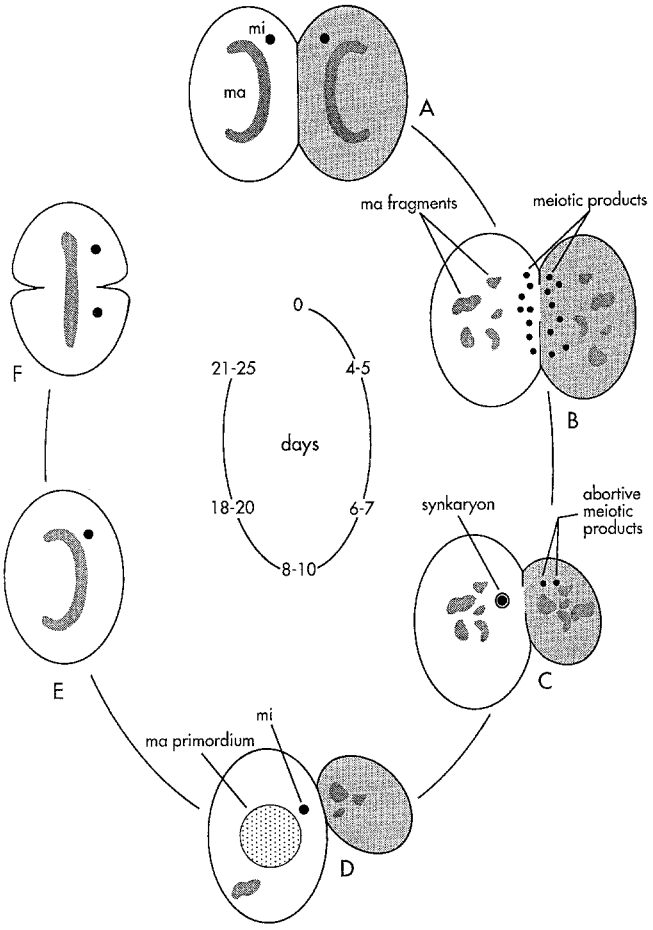

Fig. 4. Diagrammatic representation of some nuclear stages in mating and post-mating cells of *E. focardii*. Light and shadowed cytoplasms indicate cells of Mt's I (strain TM_4) and II (strain TM_{15}), respectively. *Stage A*, initial pair between apparently identical vegetative cells, both with one spherical micronucleus (mi) and a C-shaped macronucleus (ma); *stage B*, 4-5 day old pair in which both mates completed the third pregamic division and contain eight haploid nuclei; *stage C*, 6–7 day old pair in which only one mate formed the synkaryon; *stage D*, 8–10 day old pair in which only one ex-conjugant underwent post-mating reorganization and formed both the primordium of the new macronucleus and a new micronucleus; *stage E*, ex-conjugant with a completely reconstituted nuclear apparatus; *stage F*, first division of the cell reorganized from mating

The death or survival of only one of the two partners of a mating pair is a rather unusual phenomenon in free-swimming (non sessile) ciliates. A prerequisite to approach the biological meaning of this phenomenon is knowledge of whether or not the mating cell death or survival is cell-type specific. With the aim of investigating this question, aliquots of Mt-I and Mt-II cell cultures were alternately labeled before being mixed with the other unlabeled ones. Labeling was carried out at the nuclear level, by a 3-day cell incubation with [^{3}H]-thymidine (for a period presumed to be long enough to permit cells to complete one cell cycle and hence pass through one S-stage of macronuclear DNA synthesis). In total, 200 mating pairs of 6–7 days of age (formed by cells clearly different from each other in size) were prepared. Of these, only 165 could be used: they appeared to be heterotypic, formed by one labeled and one unlabeled cell. The other 35 pairs were discarded due to indecisive cell labelling. In every case, the normal and the defective partners were found to be derived from Mt-I and Mt-II cell cultures, respectively, and the cell behavior in the pair was thus shown to be associated with the cell type. Additional evidence for this conclusion was derived from analyses of mixtures prepared between a morphological variant, "doublet" cells of Mt-I (which had developed spontaneously and were isolated from standard cultures of singlets) and normal singlets of Mt-II. These mixtures produced pairs in which doublets always behaved normally and singlets defectively.

Life cycle

Following a sexual event, ciliates start a new clonal life cycle, with an initial stage of immaturity followed by maturity. These stages are measured in numbers of cell fissions (rather than in calendar time) and can be operatively identified on the experimental observation that cells of a clone are initially not responsive to mating stimuli and later become responsive to them. To obtain preliminary information on the life cycle of *E. focardii*, a set of 72 sister clones was derived from 100 exconjugants isolated from a mixture of Mt-I and Mt-II cells, and 30 of these clones were analyzed at weekly intervals for the multiplication rate and the development of the mating competence (as shown by mixing samples with both parental cell types). Up to an age of 120 fissions (equivalent to about one year), no clones were found to have reached mating competence. Subsequently, in the interval between 120 and 160 fissions, 13 clones started forming mating pairs in mixtures with Mt-II parental cells but not in mixtures with Mt-I parental cells. The other 17 clones (of the 30 examined) were never observed to mate, thus implying that their immaturity is longer than one year (or even permanent). Understandably, this long-lasting immaturity of many exconjugant clones discouraged any further analyses of the mechanism of mating type inheritance of *E. focardii*. However, it is worth noting that the finding that every mating-reactive offspring clone had the same mating type as the Mt-I parental cells is consistent with the assumption (made above) that no gene transfer occurred from Mt-II to Mt-I cells during mating.

Discussion

From the observations described above, rather unusual aspects appear to distinguish the biology of *E. focardii* from that of other *Euplotes* species. These aspects are related to the reproductive stage of the life cycle and, to a larger extent, to the sexual process of mating.

The most important constraint on the reproduction of *E. focardii* under laboratory conditions was the temperature, as also reported for another Antarctic *Euplotes* species, *E. antarcticus*, collected from the Western area of Weddell Sea and studied by Lee and Fenchel (1972). On the other hand, no particular food organisms or feeding regimens were required for *E. focardii*. Cells kept at temperatures above 10–11°C became abnormal in shape and morphology, and died subsequently. This apparently close dependence of *E. focardii*'s elementary life functions on a low environmental temperature probably reflects an ancient adaptation of this ciliate to cold waters and, also, suggests that endemic ciliate species coexist in Antarctica with more recent immigrant species. In this connection, it may be pointed out that strains representative of another *Euplotes* species, *E. rariseta* (Valbonesi and Luporini 1990b), collected together with *E. focardii* from Terra Nova Bay, have been observed to increase their multiplication rates in parallel with an increase in the environmental temperature up to 16–18°C (data not published), that is, a temperature value close to that usually chosen (20–22°C) for maintaining other *E. rariseta* strains from temperate and tropical waters in the laboratory (Dallai et al 1987).

Sexual processes in *E. focardii* were apparently restricted to the cell union of mating pairs. Like other species of *Euplotes* (as reviewed by Nobili et al 1987), regulation of this union in *E. focardii* involved a complex of different mating types. We could not, however, determine whether the Mendelian mechanism of inheritance of these mating types involves serial dominance, or co-dominance between alleles at the *mat* (mating type) locus, because of the exceptionally long-lasting immaturity of exconjugant clones. On the other hand, mating pairs of *E. focardii* showed unique developmental features in comparison with every other *Euplotes* species so far studied, where mating pairs involve a temporary union (usually lasting 12–14 h) between two cells which are functionally and morphologically indistinguishable from each other (and usually denominated as "iso-conjugants" or "isogamonts").

E. focardii mating pairs showed a dramatic change from an initial symmetric stage of union between apparently identical cells, mutually coordinated in nuclear activity, to a final asymmetric stage involving union between a macro- and a microconjugant. The former carries out regular nuclear processes and yields a new clone after recovery from conjugation; the latter is subject to cell body shrinking and is irreversibly committed to die.

Does this sharp divergence manifested by *E. focardii* from the basic model of mating of the other species of *Euplotes* reflect a specific, adaptive requirement? We are prompted to speculate that it does, and to identify this requirement with the cost that *E. focardii* must pay for exploiting sex under environmental conditions that impose an exceptionally long-lasting mating union of about 8–10 days. As this union is inescapably accompanied by absolute starvation (in fact the cytostome of a mating cell is mechanically obstructed from ingesting food), *E. focardii* has probably adopted a strategy of unidirectional, or largely preferential, mate-to-mate transfer of cytoplasm in order to supplement the recipient mate with additional resources for a more efficient cell recovery of the vegetative and reproductive stage. This view is supported by the knowledge that a wide cytoplasmic intermate connection is regularly developed by every species of *Euplotes* (Dallai and Luporini 1989, and references therein) and, as well, by some species of hypotrich ciliates, e.g., *Paraurostyla weissei* (Jerka-Dziadosz and Janus 1975), *Pseudourostyla levis* (Takahashi 1983), and *Oxytricha hymenostoma* (Banchetti et al. 1980), which perform mating by a permanent fusion of pairs of cells which ultimately share their cytoplasms in a single "synconjugant".

Finally, an intriguing aspect of *E. focardii* mating is related to the apparent mating type-specificity of the divergent behavior of the two pair members. It was shown that the type of the normal member was Mt-I and that of the abnormal one Mt-II. However, inheritance of the mating types could not be studied due to the long-lasting immaturity of exconjugant clones. In principle, it might be due either to a direct action of *E. focardii* nuclear genes, or to cytoplasmic factors, or to bacterial endosymbionts, as

is known to occur in the mate-killer phenomenon of *E. crassus* (Dini and Luporini 1982).

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