Purification and initial characterization of two pheromones from the marine Antarctic ciliate, Euplotes nobilii

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ABSTRACT

Among a set of wild-type strains of *Euplotes nobilii*, every one derived asexually from one specimen isolated from Terra Nova Bay (Ross Sea, Antarctica), two were found to be representative of different mating types mutually capable of inducing each other to form mating pairs through pheromones constitutively secreted into the extracellular environment. Pheromones of strain AC-1 were purified to homogeneity and shown to be represented by two distinct proteins, that were denoted E*n*-1 and E*n*-2. E*n*-1, secreted in amounts three-fold greater than E*n*-2, was determined to have a molecular weight of 5617 and an asparagine at the N-terminus of its amino acid sequence, while E*n*-2 has a molecular weight of 6290 and bears an asparctic acid at its N-terminus. The fact that E*n*-1 and E*n*-2 are coreleased by genetically identical cells of the same strain was taken to imply that they carry a heterozygotic combination of allelic pheromone genes and that these genes are regulated by relationships of co-dominance.

KEY WORDS: Protein cell-markers - Ciliate mating-types - Cell recognition - Protozoa.

INTRODUCTION

The production of molecular markers for the purpose of *self-nonself* discrimination among cells of the same organism is a property usually assumed to be unique to multicellular forms of life. In effect, this production can already be observed in ciliates in association with their mating type systems (Luporini & Miceli, 1986; Miyake, 1996), which in some species evolved for controlling the cell shift from the asexual stage of vegetative (mitotic) reproduction to the sexual one of conjugation (or mating) (Dini & Nyberg, 1993). The existence in ciliates of such markers, now usually denoted as pheromones and earlier as mating-type factors or gamones, can readily be appreciated by mixing together in various pairwise combinations conspecific strains which developed vegetatively from single parental cells. Cells will form mating pairs only in certain mixtures, but not in others, thus implying that they can recognize different marker molecules distinctive of different mating types in the former case, and that they share identical markers and the same mating type in the latter.

The chemical nature of ciliate pheromones has so far remained elusive in ciliates such as *Paramecium* and *Tetrahymena* that have usually been taken as major reference systems in "Experimental Ciliatology" (Nanney, 1980), in particular for studies on the genetics and biology of the mating type systems. The nature of these molecules has been determined in other ciliates, *Blepharisma*, *Dileptus*, and *Euplotes*, in which limited (*Dileptus*) or no information at all (*Blepharisma*) exists on the mechanism of mating type control and inheritance. Only in *Euplotes* has it been shown that the mating types and their associated pheromones are controlled by multiple series of alleles, which are inherited at an apparently single genetic locus, i.e., the *mat* or mating-type locus, with relationships of serial dominance in some species, or co-dominance in others (Nobili *et al.*, 1987; Dini & Nyberg, 1993).

Pursuing pheromone purification and characterization has been more successful in *Blepharisma*, *Dileptus*, and *Euplotes*, because these ciliates secrete and diffuse their pheromones constitutively in biologically detectable concentrations into the extracellular environment, unlike *Paramecium* and *Tetrahymena* which apparently retain these molecules bound to the cell surface (Hiwatashi Kitamura, 1985; Kitamura, 1988; Wolfe, 1993; Driscoll & Hufnagel, 1999). In the environment, pheromones have usually been revealed by mating-induction assays in which the formation of mating pairs is induced after suspension of a cell culture with cell-free supernatant of another culture of different mating type. Otherwise, they can be revealed by assays based on their less appreciated, yet most likely primary, activity as mitogenic factors (Parfenova *et al.*, 1989; Vallesi *et al.*, 1995).

Only one case has been reported in which pheromones of the same species are represented by chemical-

ly unrelated molecules. In *B. japonicum*, one pheromone has in fact been determined to be a glycoprotein of about 20 kDa (Miyake & Beyer, 1974), and the other to be a 3-(2'-formylamino-5'-hydroxybenzoyl)lactate (Kubota et al., 1973), possibly a tryptophan derivative and a serotonin-related molecule (Jaenicke, 1984). In the other ciliates in which pheromones have been isolated, i.e., *E. raikovi* (Miceli et al., 1983; Vallesi et al., 1996), *E. octocarinatus* (Weischer et al., 1985; Schulze Dieckhoff et al., 1987; Mollenbeck & Heckmann, 1999), *E. patella* (Akada, 1985), and *D. anser* (Parfenova et al., 1989), they have all been found to be represented by relatively small proteins in the range from circa 3 to 20 kDa. In addition, in *E. octocarinatus* (Brunen-Nieweler et al., 1998) and *E. raikovi* (Raffioni et al., 1992; Vallesi et al., 1996), in which the amino acid sequences and (limitedly to *E. raikovi*) the three-dimensional structures of several of these proteins have been resolved (Luginbuhl et al., 1994; Weiss et al., 1995), it appears evident that they represent species-specific families of homologous molecules, in full accord with their genetic control through multiple *mat* alleles.

In this study, we present data on the identification and purification of two pheromones of another species of *Euplotes*, *E. nobilii*, that has been collected, and described as new, from marine sandy bottoms of Terra Nova Bay in Antarctica (Valbonesi & Luporini, 1990). A structural and biological characterization of these new pheromones in addition to being significant in an ecological perspective in relation to the Antarctic distribution of *E. nobilii*, may also contribute new insights into the taxonomic relationships among *Euplotes* species. It has in fact been recently proposed that the genus *Euplotes* should be split into four genera, i.e., *Euplotes*, *Euplotoides*, *Euplotopsis*, and *Moneuplotes*, on the basis of divergences in number and arrangement of ventral ciliary organelles (cirri) and shape of the argentophilic layer (argyrome) that is generated by the juxtaposition of cortical alveoli underneath the plasma membrane on the cell dorsal surface (Borror & Hil, 1995). Since *E. nobilii* has been re-assigned to *Euplotes* and is separated from both *E. octocarinatus* and *E. raikovi* (classified as *Euplotoides* and *Euplotopsis*, respectively), a structural comparison of the pheromones of these three species may provide additional criteria for validating this new taxonomic picture of euplotes.

MATERIALS AND METHODS

Strains, culture conditions, and preparation of cell-free supernatant

Strains AC-1 and AC-4 were used: the former as pheromone source, the latter for bioassaying pheromone activity. Their cultures were maintained in a cold room, at 4° C, exposed to a rhythm of 16 h of darkness and 6 h of very weak light, and fed on green algae *Dunaliella tertiolecta*. These were grown in natural seawater sterilized and added with Walne medium before being used. Volumes of cell-free supernatant for pheromone purification were obtained from cultures in which cells were allowed to multiply for about 1 week in presence of plenty of food, and then concentrated

and suspended for 3-4 days, at a density of $1-2 \times 10^4$/ml, in fresh seawater without food. Supernatant preparations were passed first through paper filters to remove gross debris in suspension and then through filters of 0.2-μm pore size to remove other particulate material, before being used for pheromone extraction.

Materials and reagents

The following material and reagents were purchased: Sep-Pak C_{18} cartridges from Waters Corporation (Milford, MA); Superose 12 HR 10/30 and Mono Q HR 5/5 from Pharmacia Biotech (Piscataway, NJ); Bio-Rad Protein Assay Kit II from Bio-Rad Laboratories (Hercules, CA); tris, NaCl, and 2-propanol from Merck (Darmstadt, Germany); sodium borate, CHAPS, and standard molecular weights from Sigma-Aldrich (St. Louis, MO).

Chromatography

Sep-Pak C_{18} cartridges were used for pheromone adsorption after treatment with 2 ml of pure 2-propanol and 5 ml of double-distilled water. Loaded cartridges were eluted with 2 ml of 30% (v/v) 2-propanol, and the eluted material was dried in a Speed Vac concentrator (Savant, Farmingdale, NY). Unless immediately used, dried material was pooled and stored at -20° C. The Superose 12 HR 10/30 column used in size-exclusion chromatography was equilibrated in 50 mM tris, pH 7.8, and run at a constant flow rate of 0.5 ml/min. The Mono Q HR 5/5 column used in ion-exchange chromatography was equilibrated with tris 20 mM, pH 7.5, and eluted with a 0-0.4 M NaCl linear gradient in the same buffer, at a constant flow rate of 0.5 ml/min.

Capillary electrophoresis

Capillary electrophoresis was performed on a BioFocus 3000 apparatus (Bio-Rad Laboratories), using an uncoated bare silica capillary (50 cm long × 50 μm I. D.) conditioned and filled with 0.2 M sodium borate, pH 9.0, and containing 0.5% CHAPS. Samples were suspended and injected in 10 mM sodium borate, pH 9.0, at a final concentration of 0.1 mg/μl. Separation was obtained by applying a constant voltage of 18 kV, for 25 min, at 18° C, and monitoring UV absorbance at 200 nm.

Mass spectrometry

Mass spectral analysis was performed in PRIMM laboratories (San Raffaele Biomedical Science Park, Milano, Italy), using a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer (Voyager-DE Biospectrometry Workstation model from PerSeptive Biosystems, Framingham, MA).

RESULTS AND DISCUSSION

Mating types and pheromone secretion

In a search for the existence of different and compatible mating types among the wild-type strains previously classified as *E. nobilii* on the basis of traditional morphological criteria (Valbonesi & Luporini, 1990), cultures of these strains were mixed together in every pairwise combination after having been grown under conditions favouring manifestation of their mating competence, that in ciliates usually ensues in coincidence with a shift of a cell culture from growing to starving conditions. Mating pairs did form in numerous of these mixtures, thus suggesting that at least some strains among the dozen analysed were representative of different and mutually compatible mating types. Strain AC-1 was chosen to represent one mating type, and strain AC-4 to

represent a different and mating compatible one. Accounting for the choice of these strains was not only the fact that they showed a mating reactivity appreciably higher than any other strain combination; another reason was that pairs formed by these mixtures were heterotypic, i.e., between one AC-1 cell and one AC-4 cell, as well as homotypic, or "illegitimate", i.e., between cells from the same strain. This latter observation (carried out in some mixtures prepared with cell lines previously labeled in order to distinguish one from the other) represented a condition very favourable (probably, necessary) for assaying pheromone secretion in strains AC-1 or AC-4, utilizing the usual procedure of monitoring the formation of (homotypic) mating pairs between cells of one strain suspended with supernatant from cultures of another strain of different mating type.

Tu pursue this intent, cells and preparations of cell-free supernatant were recovered from mating competent cultures of both strains AC-1 and AC-4, and then used to suspend cells of one with supernatant of the other and *vice versa*. In both combinations, cells formed mating pairs usually 10-15 h after having been suspensed with the heterologous supernatant, thus denoting the presence of a "foreign" (i.e., different from their own) pheromone in their new environment. Although both combinations yielded mating pairs, the response of AC-4 cells was appreciably stronger than that of AC-1 cells (as evaluated by counting percents of cells united in pairs over the total mixed and percents of pairs capable of completing conjugation over pairs intially formed). Thus, based on a simplified rationale intrinsic with these observations, i.e., the stronger the mating-inducing activity of a cell-free supernatant, the higher the amounts of pheromone it contains, it was decided to use strain AC-1 for pursuing pheromone purification, while strain AC-4 provided tester cells for assaying pheromone activity. In these assays, pheromone activity was measured with "arbitrary units" established as the minimal amount required by a pheromone preparation for inducing formation of at least one mating pair in 1-ml sample of about 10^3 tester cells. This criterion for measuring pheromone activity was originally established in studies of *Blepharisma* pheromones (Miyake & Beyer, 1973) and, for the sake of uniformity, has subsequently been adopted for monitoring and comparing pheromone activity also in other ciliates. The reader is however advised that its application, although simple and quick, holds only for the paracrine-like activity that a pheromone exerts as mating-inducing factor on cells that are not the same as those from which it is produced; it disregards the autocrine activity that the same pheromone may exert as a mitogenic factor on the same cells from which it is synthesized (Vallesi *et al.*, 1995). In addition, the measurements deduced from the application of this criterion may greatly vary, even by three orders of magnitude or more (i.e., from 10^{-9} to 10^{-12} M) according to whether tester cells are used in the presence of their secreted pheromone in the medium, or af-

ter having been washed with fresh medium and their pheromone removed (Ortenzi & Luporini, 1995).

Pheromone purification

To carry out pheromone purification from strain AC-1, a protocol essentially identical to the one originally devised for purification of *E. raikovi* pheromones (Concetti *et al.*, 1986) was utilized. It involves three basic steps: (i) pheromone adsorption onto Sep-Pak C_{18} reverse-phase cartridges, (ii) gel-filtration chromatography on Superose-12, and (iii) ion-exchange chromatography on a Mono Q column. Typically, two-liter preparations of cell-free medium recovered from cultures of strain AC-1 were each passed through a Sep-Pak C_{18} cartridge, with an apparently complete recovery of pheromone activity and adsorption of relatively few contaminants. These were removed in large measure through the successive purification step on Superose-12 chromatography, that generated an elution profile in which protein and pheromone activity coexisted in a set of fractions (28-40) of a peak emerging at about 8 kDa (Fig. 1). These fractions were then pooled and the protein pool applied onto a Mono Q column. Shown in Figure 2 is the elution profile from this column revealing two sharp peaks, clearly separated from one another and of unequal size, peak 1 being markedly wider than peak 2.

These results were thus taken as evidence that two distinct proteins coexisted in eccentric ratio in the cell-free medium of strain AC-1, and that their separation into two homogeneous pools (one derived from mixing together fractions 35-39 of peak 1 eluted with 0.22 M NaCl, and the other from mixing together fractions 41-45 of peak 2 eluted with 0.25 M NaCl) had effectively been achieved through the last purification step on ion-exchange chromatography. Both protein pools proved to be capable, at a concentration of the order of 10^{-8} M, of inducing mating pair formation between cells of strain AC-4 (used without previous suspension in fresh medium, and hence in the presence of their secreted pheromone). Consistently with this activity, they were designated to represent two distinct pheromones of *E. nobilii* (hereafter denoted as En-1 and En-2) and assumed to be specified by two distinct genes allelic and co-dominant at the *mat* locus, such as is the case in *E. raikovi* (Luporini *et al.*, 1986), *E. patella* (Akada, 1986), and *E. octocarinatus* (Weischer, 1985, in Schulze Dieckhoff *et al.*, 1987). After having been assayed for homogeneity on a capillary zone electrophoresis system (Fig. 3), the final preparations of En-1 and En-2 were quantitated and analysed for determinations of molecular weight and amino acid sequence.

Table I illustrates quantitative data of a typical purification of En-1 and En-2, carried out from a starting volume of 10 liters of cell-free medium and by measuring protein concentration at every purification step according to Bradford's method (1976). It appears that 150 mg of apparently homogeneous protein were purified for En-1 and 50 µg for En-2, equivalent to a ratio of 3:1
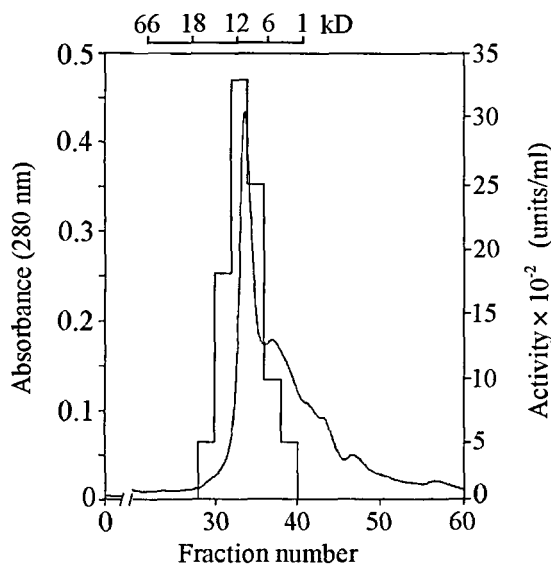
Fig. 1 - Size-exclusion chromatography on a Superose-12 column. The applied sample is material eluted from Sep-Pak C_{18} cartridges used for reverse-phase chromatography of 10 liters of cell-free medium of strain AC-1. Pheromone activity, assayed every second fraction, is indicated by light columns. For calibration of the column molecular standard weights are reported in kD.
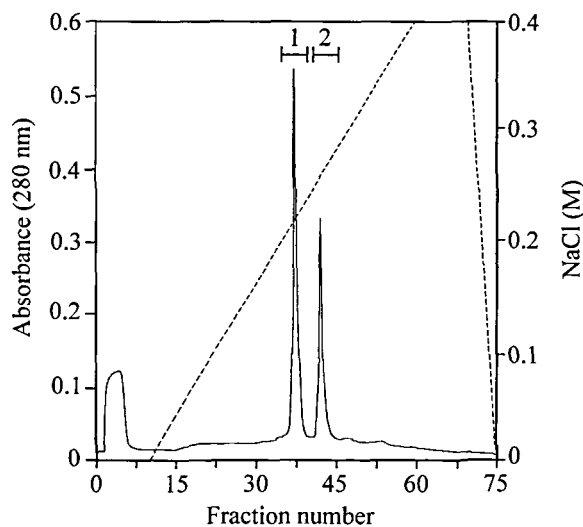

Fig. 2 - Ion-exchange chromatography on Mono Q HR 5/5 column. The applied sample (220 µg of protein in 2 ml of 20 mM tris, pH 7.5) is material from Superose-12 column. Gradient of NaCl used for elution is indicated by the broken line. Pheromone activity, assayed every second fraction, is indicated by segments 1 and 2 overlying the two peaks.

Fig. 3 - Capillary zone electrophoresis for analysis of purity of E*n*-1 and E*n*-2. The applied samples are protein preparations associated with peaks 1 and 2 eluted from ion-exchange chromatography, panels A and B, respectively. Electropherograms show a single and sharp peak demonstrative of the sample homogeneity.

(E*n*-1: E*n*-2). With regard to determinations of molecular weight and amino acid sequence, samples of E*n*-1 and E*n*-2 were analyzed under native conditions in mass spectrometry and subjected, after carboxymethylation, to automated sequential phenylisothiocyanate (Edman) degradation (kindly performed by Dr. Vito Carratore, Institute of Biochemistry of Proteins and Enzymology of C.N.R., Naples). Mass spectral analysis permitted,
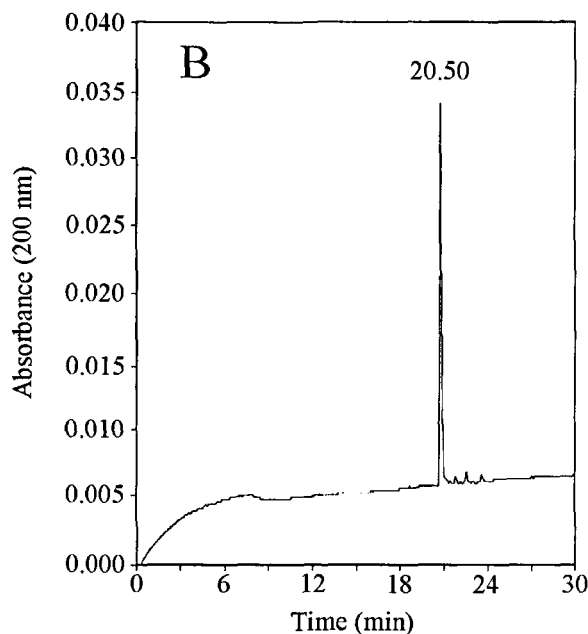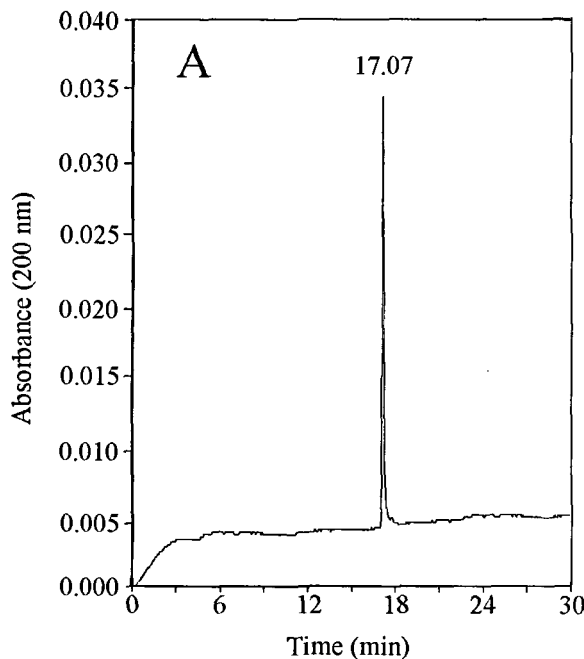
as shown in Figure 4, a conclusive assignment of a molecular mass of 5617 to E*n*-1 and of 6290 to E*n*-2, which are values consistent with amino acid sequences of 51 and 57 residues, respectively. Chemical analysis revealed that the N-terminus of E*n*-1 amino acid sequence is formed by the strand Asn-Pro-Glu-Asp-Trp, while the N-terminus of E*n*-2 sequence bears a strand which is different in three positions, Asp-Ile-Glu-Asp-Phe.
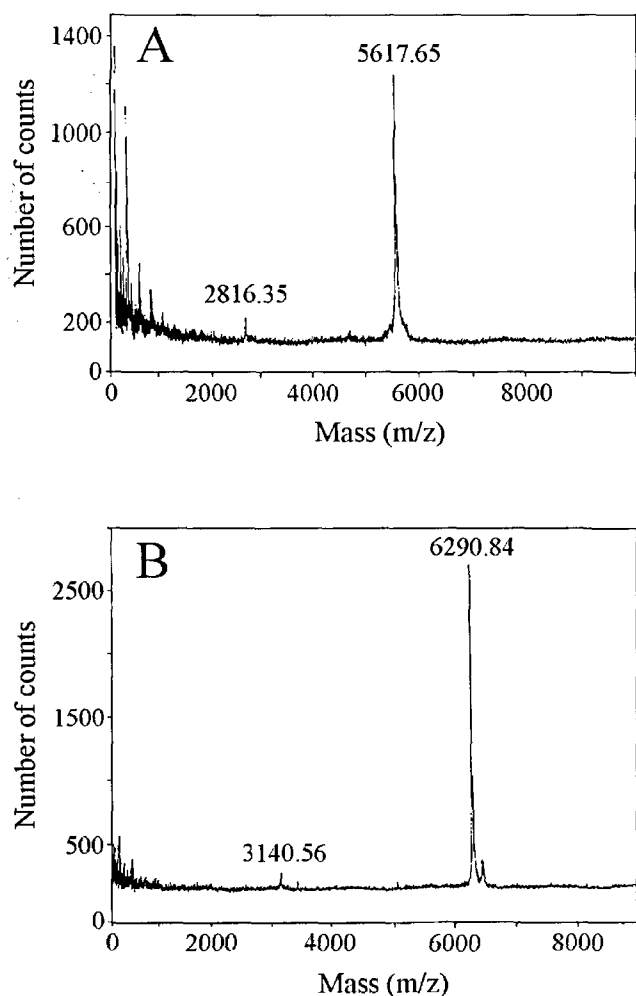
Fig. 4 - Mass spectrometric analysis of E*n*-1 and E*n*-2. Spectra are shown only in the m/z (mass-to-charge ratio of the molecular species) region up to 8000. Panel A, mass measured for E*n*-1; panel B, mass measured for E*n*-2.

Conclusions

In conclusion, (i) we have identified strains of *E. nobilii* that secrete their pheromones constitutively, (ii) purified to apparent homogeneity two of these pheromones, *En*-1 and *En*-2, from the cell-free medium of

cultures of strain AC-1, and (iii) shown that *En*-1 and *En*-2 are represented by two proteins that differ from one another in molecular weight, amino acid sequence at the N-terminus, and amounts in which they appear to be synthesized. Since the determination of the complete amino acid sequence of these proteins is in progress, it seems appropriate to wait for the completion of this study before proposing a more insightful discussion on the significance of similarities and differences between *En*-1 and *En*-2 and other *Euplotes* pheromones that have so far been structurally characterized. As matters stand, our results hold essentially in a general context of *Euplotes* biology. They suggest that a manifest pheromone secretion is a phenomenon that *Euplotes* species exploit independently of the habitat they have colonized, be this habitat represented by sea waters of Antarctica (*E. nobilii*), of temperate continents (*E. raikovi*), or represented by freshwater ponds (*E. patella, E. octocarinatus*). Rather, it appears to be a feature common to a larger group of *Euplotes* species, such as all the four mentioned above, that control their multiple mating types and their associated pheromones through genes regulated by relationships of codominance. In effect, in the less represented species of *Euplotes* in which these genes are expressed with relationships of serial dominance, i.e., those species which were earlier recognized as the *crassus-minuta-vannus* species complex (Nobili *et al.*, 1987) and have now been assigned to the *Moneuplotes vannus* group in the new taxonomic scheme of *Euplotes* by Borror & Hill (1995), pheromones have not been detected in the culture medium by the usual assays of mating induction, and their activity has been assumed to be mediated through physical cell-cell contacts (Miyake & Nobili, 1974; Dini & Miyake, 1982).

It is a common view to consider the former, larger group of species to be evolved from the latter one, the former being endowed with more complex, 'double' or 'multiple', argyromes (i.e., with two or more lines of cortical alveoli between two adjacent ciliary rows on the cell dorsal surface) and the latter with 'single' argyromes (Borror & Hill, 1995; Valbonesi & Luporini, 1995). Do these evolutionary relationships among *Euplotes* species also imply that pheromone secretion represents a secondary acquisition of the life history of *Euplotes*? It has been

TABLE I - *Summary of a tipical purification of pheromones En-1 and En-2 from 10 liters of cell-free medium of strain AC-1 of* Euplotes nobilii.

Stage	Pheromones	Total protein (µg)	Total activity (units × 10^{-2})	Specific activity (units/µg × 10^{-2})	Purification (-fold)	Yield (%)
Cell-free medium	E*n*-1 + E*n*-2	310	100	32	1	100
1. Sep-Pak C$_{18}$ cartridges	E*n*-1 + E*n*-2	280	92	33	1.03	92
2. Superose-12 chromatography	E*n*-1 + E*n*-2	220	88	40	1.25	88
3. Mono Q chromatography	E*n*-1	150	63	43	1.34	63
	E*n*-2	50	22	44	1.37	22

found that *E. raikovi* (one of the species with complex argyromes) in addition to synthesizing pheromones in their diffusible (standard) form, also synthesizes an isoform that remains anchored to the cell surface, where it may assist cells in signaling through direct cell-cell contacts (Miceli *et al.*, 1992). A mechanism of alternative splicing of the primary trascripts of the pheromone genes is responsible for this synthesis, that in practice enables cells using their pheromones for both medium-mediated (long-range) and cell-cell contact-mediated (short-range) signaling. A less efficient, more primitive version of this mechanism, still incapable of ensuring a balanced production of the two pheromone forms and thus directed, in a much larger measure, to sustaining the synthesis of pheromones in their cell-bound isoform, could well account for the apparent lack of pheromone secretion in the species of the *Moneuplotes vannus* group.

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