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Cellular Mechanisms of the Evolution of Ontogenesis in *Volvox*

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Key words: Cell division; Evolution; Ontogenesis; *Volvox*

Summary

The green flagellates of the genus *Volvox* can be conditionally subdivided into two groups according to the size of gonidia (asexual reproductive cells) at the onset of cleavage. In *Volvox carteri* and several other species the gonidium undergoes an extended period of hypertrophied growth, after which a series of rapid fissions occurs. Embryonic cells do not grow during the intervals between consecutive divisions. In representatives of the second group (e. g., *V. aureus*) the period of gonidial enlargement is comparatively nondurable; thus the cleavage begins when the gonidium is relatively small, and each division is followed by a period of cellular growth.

In this paper the evolutionary relationships between two types of asexual life cycle in *Volvox* are analysed on the basis of literary and our own data. It is supposed that the *V. aureus* type of development with slow divisions of small gonidia is more advanced in the evolutionary respect than the *V. carteri* type of development with rapid divisions of large gonidia.

Experimental analysis of the role of light and dark for embryonic cleavage progression in *V. aureus*, *V. carteri* f. *nagariensis* and *V. tertius* as well as the experiments with several metabolic inhibitors (aminopterin, actinomycin D, cycloheximide and streptomycin) have enabled us to elucidate cellular mechanisms of the evolution of ontogenesis in *Volvox*.

1. Introduction

The green flagellate *Volvox* offers an opportunity to study development in a relatively simple system consisting of two kinds of cells, somatic and reproductive. The asexual individual of *Volvox* is a spheroid in which several hundred or thousand cells are arranged in a single peripheral layer and are embedded in a transparent extracellular matrix. Biflagellate somatic cells, similar in structure to *Chlamydomonas*, make up the bulk of the organism. They propel the spheroid of *Volvox* through the medium and synthesize most of the extracellular glycoproteinaceous material; these cells fail to divide but instead they undergo terminal differentiation, programmed senescence and death. Cells of the second type, the non-flagellated gonidia, occur in small numbers (usually no more than 8–16) and are located in the posterior region of the spheroid. During asexual reproduction these gonidia at first enlarge and later divide to form new individuals. *V. carteri* f. *nagariensis* has proved to be the most suitable representative of the genus *Volvox* for the experimental analysis of cellular differentiation control. Thus the latter species and forma of *Volvox* can be used as a valuable developmental model (reviews: JAENICKE & GILLES 1985; KIRK & HARPER 1986).

On the other hand, *Volvox* and some other colonial green flagellates belonging to the family Volvocaceae have traditionally attracted the attention of evolutionary biologists. It is known for a long time that the genera *Gonium*, *Pandorina*, *Eudorina*, *Pleodorina* and *Volvox* form an interesting series of organisms showing an increase in cell numbers, in the complexity of morphogenesis and in the differentiation between somatic cells and reproductive cells. In an attempt to elucidate the genetic aspects of the origin of multicellularity in this series, KIRK

(1988) has recently analysed the phylogenetic role of three categories of mutations that cause a breakdown of the division of labour between somatic cells and gonidia in *V. carteri* f. *nagariensis*. He has supposed that the appearance of three loci (regA, gls and lag) in the Volvocalean genome might be a prerequisite for the evolutionary transition from a colonial state (in which all cells are totipotent) to the truly multicellular state with the irreversible division of labour between two fully differentiated cell types.

This paper is also devoted to an analysis of the evolutionary aspects of ontogenesis. The main emphasis, however, is made in the field of a comparison of specific traits of the asexual development in various species of *Volvox*. Therefore, at first the taxonomy of the genus *Volvox* is briefly considered. Then a survey is given of literary and our own data concerning the control of gonidial divisions in *V. aureus*, *V. carteri* and *V. tertius*. In conclusion, an attempt is made to elucidate cellular mechanisms of the evolution of asexual ontogenesis in *Volvox*.

2. Material and Methods

Volvox carteri f. *nagariensis* IYENGAR (some zygotes made by crossing the female strain HK-10 and the male strain 69-1b) was kindly supplied by Professor R. C. STARR (University of Texas at Austin). We germinated the zygotes, recovered the male and female strains from the progeny and used the female strain in our work. Clonal cultures of *V. aureus* EHR. and *V. tertius* MEYER (the homothallic strains P-1 and V-3 respectively) originated from the material found in Leningrad region. The algae were kept in soil water medium containing CaCO₃ (STARR 1964) and grown axenically in standard *Volvox* medium (STARR 1969) adjusted to pH 8 under a light/dark regime of 16 h-8 h at 22-24 °C. The illumination with an intensity of approximately 2000 lux was provided by cool white fluorescent tubes. The cultures were routinely transferred twice a month to 5 or 10 ml of fresh media (in test-tubes or small flasks) to ensure the active asexual reproduction. Synchronous and semi-synchronous cultures were started with a single spheroid in 5-25 ml of fresh medium. Under the regime used in this work, the algae tend to grow synchronously over several generations with an asexual life cycle of 4-5 days. To determine the diurnal rhythms of divisions, the cultures with a density of 30-60 spheroids per ml were checked every one or two hours during the days when the majority of embryos underwent cleavage. To determine the role of light for the proceeding of cleavage, the material was placed in the dark for 12-20 h. It should be noted that using soil water and standard *Volvox* medium gave the same results concerning the rhythms and the light/dark control of cell divisions.

To examine the effect of inhibitors on *Volvox* development, the solutions of aminopterin (Fluka, Switzerland, actinomycin D (Serva, FRG), cycloheximide (Serva, FRG) and streptomycin (Minmedprom, GUS) were prepared in standard *Volvox* medium. All experiments were repeated twice. In every experiment 5-10 intact spheroids with 40-80 embryos at 2-8-celled stages of cleavage were used. The material was taken from mass cultures by means of a glass micropipette and the number of cells in each embryo was scored under the microscope. Then these spheroids were placed into test-tubes containing 1-3 ml of a solution of an inhibitor at any given concentration for 20-24 h (*V. aureus* and *V. tertius*) or 18 h (*V. carteri* f. *nagariensis*) at continuous light of 2000 lux (24-26 °C). In the beginning of all experiments using light-sensitive aminopterin, treated spheroids were transferred to darkness for 4 h prior to illumination for subsequent development. At the end of each experiment, the spheroids were placed again under the microscope and the numbers of cells were scored once more. The mean number of cell divisions completed in a solution of an inhibitor was calculated by a formula:

$$n = \frac{\ln N_2 - \ln N_1}{\ln 2}$$

where N_1 and N_2 are the total numbers of cells in all 40-80 embryos at the beginning and at the end of every experiment respectively. It should be noted that the control, non-treated embryos of *V. aureus* and *V. tertius*, underwent 5-6 and 7-8 further divisions respectively in the course of 20-24 h of continuous illumination. In the control embryos of *V. carteri* f. *nagariensis* the whole process of cleavage (a series of 11-12 divisions) and inversion were already completed within 18 h.

3. The taxonomy of the genus *Volvox* (L.) EHR.

According to SMITH's (1944) survey, the genus *Volvox* includes 18 species. It can be divided into four taxonomic sections which are distinguished by: 1. the presence or the absence of cytoplasmic bridges between adjacent protoplasts (in the former case the thickness of the cytoplasmic connections is an important criterion as well), 2. the size of gonidia at the onset of cleavage, 3. the shape of somatic cells and 4. the structure of the extracellular matrix. This classification is used currently, though previous data on the matrix structure (SMITH 1944) have been recently made more exact (KIRK et al. 1986). The list of the sections and species of *Volvox* which is given below differs slightly from that in SMITH's survey: a new species, *V. pocockiae* (STARR 1970a), is added, and another species, *V. weismannia* POWERS, is excluded; the latter is now regarded as *V. carteri* f. *weismannia* (POWERS) IYENGAR (KOCHERT 1975; NOZAKI 1988).

The section Merrillosphaera (SHAW) PRINTZ includes *V. africanus* WEST, *V. carteri* STEIN, *V. gigas* POCOCK, *V. obversus* (SHAW) PRINTZ, *V. powersii* (SHAW) PRINTZ, *V. spermato-sphaera* POWERS and *V. tertius* MEYER. All members of the section are characterized by the lack of cytoplasmic connections between the protoplasts. Gonidia reach relatively large size before the onset of cleavage; their diameter is more than 30 μm .

The section Janetosphaera (SHAW) PRINTZ includes two species with thin cytoplasmic bridges: *V. aureus* EHR. and *V. pocockiae* STARR. The former is characterized by the mature gonidia of relatively small diameter (up to 18–25 μm), whereas in the latter gonidia reach a maximum of 60 μm in diameter prior to their cleavage to form embryos. In this respect the asexual reproduction in *V. pocockiae* is similar to that in the section Merrillosphaera. The structure of the extracellular matrix of *V. pocockiae*, however, is identical to that of *V. aureus*. The last fact (as well as the data on the cytoplasmic bridges) was a decisive argument for the description of *V. pocockiae* as the second species in the section Janetosphaera (see: STARR 1970a).

The section Copelandosphaera (SHAW) PRINTZ comprises the only species, *V. dissipatrix* (SHAW) PRINTZ. It is characterized by very thin cytoplasmic bridges; the gonidia reach a maximum of 15–20 μm in diameter. The matrix structure in *V. dissipatrix* differs sharply from that in the species belonging to the section Janetosphaera.

The section Euvolvox PRINTZ includes *V. amboensis* RICH et POCOCK, *V. barberi* SHAW, *V. capensis* RICH et POCOCK, *V. globator* (L.) EHR., *V. merrillii* SHAW, *V. perglobator* POWERS, *V. prolificus* IYENGAR and *V. rousseletii* WEST. All members of the section are characterized by the mature gonidia of small diameter (up to 15 μm) and by broad cytoplasmic connections between the protoplasts which have a stellate appearance in polar view (in the other three sections of the genus *Volvox* the protoplasts are nearly spherical in shape).

It is necessary to consider briefly the evolutionary relationships among various species of *Volvox*. Some ideas concerning the phylogenetic position of several members of the section Merrillosphaera have been published. *V. powersii* and *V. gigas* seem to be the most primitive species, both having many characteristics of structure and development in common with the genus *Pleodorina* (see: CAVE & POCOCK 1951; VANDE BERG & STARR 1971). On the contrary, *V. carteri* and *V. obversus* are considered to be advanced members of *Volvox*, since both species demonstrate unique characters in the process of gonidial differentiation (KARN et al. 1974).

The phylogenetic relationships among various sections of the genus *Volvox* are much less easily elucidated. In this connection it is appropriate to remind that a comparative analysis of the flagellar apparatuses in *V. carteri* f. *weismannia* (Merrillosphaera section) and *V. rousseletii* (Euvolvox section) has assumed the possibility of a polyphyletic origin of *Volvox* (HOOPS 1984). In a contemporary evolutionary protistology the sequence analysis of small-subunit ribosomal RNA is widely applied. Using this approach, RAUSCH et al. (1989) have

recently demonstrated the close phylogenetic relationship between *V. carteri* f. *nagariensis* and *Chlamydomonas reinhardtii*. Unfortunately, there are no data on ribosomal RNA sequence analysis in other species of *Volvox*.

4. Cell division controls during asexual development in various species of *Volvox*

The asexual life cycle of *Volvox* lasts several days (depending on temperature, the intensity of light and other conditions of cultivation) and includes gonidial growth, cleavage and inversion of the embryos, enlargement of young organisms while still in the parental individual, their release from the parent, further enlargement of young spheroids, etc. However, there are two principal types of asexual development in the genus of *Volvox* (STARR 1970b; KOCHERT 1975). In *V. carteri*, *V. gigas*, *V. tertius* and all other members of the section Merrillosphaera as well as in *V. pocockiae* (belonging to Janetosphaera section) the gonidia reach relatively large size prior to their divisions to form embryos (see paragraph 3). Then the extended period of cellular enlargement is followed by the process of embryonic cleavage with no growth. An interval between two consecutive divisions in the *V. carteri* embryo lasts 50–60 minutes at 20–30 °C. In the second type (*V. aureus*, *V. dissipatrix* and all members of the section Euvolvox), the mature gonidia are relatively small. The embryonic cells grow during the intervals between consecutive divisions.

The biochemical investigations of asexual life cycles in two species, *V. carteri* and *V. aureus*, are in accord with the characters of two different types of development. In *V. carteri* RNA and proteins are most actively synthesized in the gonidia during the process of their growth, but during the series of rapid divisions both transcription and translation are considerably depressed in the embryonic cells (KOCHERT 1975; YATES & KOCHERT 1976). On the contrary, in *V. aureus* RNA synthesis is minimal in the course of gonidial growth and maximal during the process of embryonic cleavage (TUCKER & DARDEN 1972). It is appropriate to note that, according to the microspectrofluorometric analysis (COLEMAN & MAGUIRE 1982), nuclear DNA replication in both species occurs only during the intervals between consecutive divisions of the embryos: each mitosis is preceded by the doubling of nuclear DNA amount.

Our analysis of the role of light and dark for cell divisions in the embryos of *V. aureus* and *V. carteri* f. *nagariensis* (DESNITSKI 1984, 1985a) correlates with the data cited above. It has been shown that in *V. carteri* f. *nagariensis*, when cultured on diurnal cycle of 16 h light – 8 h darkness (2000 lux, 22–24 °C), embryonic cleavage begins only during the restricted part of the second half of the light period: between 9 and 11 h after switching on the light. When the cleavage already began, the light is not required to support its proceeding after 2-celled stage. In darkness the rate of cleavage is as rapid as that in control light conditions: an interval between two consecutive divisions occupies about one hour. On the contrary, in *V. aureus* the cleavage starts during the first half of the light period and proceeds slowly: the interval between two consecutive divisions occupies about 4 h. The placing of the spheroids with 2–4-celled embryos in darkness blocks utterly subsequent cell divisions.

In the same papers (DESNITSKI 1984, 1985a) we have published some new data on the asexual development of *V. tertius*, a rare and poorly investigated species. The interval between two consecutive divisions in *V. tertius* embryo occupies about 3 h. The light is required for the proceeding of embryonic cleavage. The series of divisions starts in the beginning of the light period. Thus, in spite of the large gonidia which cleave without cellular growth (like *V. carteri*), *V. tertius* is similar to *V. aureus* in respect of the light/dark control and the rate of cell divisions.

An analysis of the effects of metabolic inhibitors on the embryonic cleavage proceeding in various species of *Volvox* is of great interest. Such investigations were accomplished using

V. aureus (TUCKER & DARDEN 1972), *V. carteri* (KOCHERT 1975; MARGOLIS-KAZAN & BLAMIRE 1979; WEINHEIMER 1983) and *V. tertius* (IRELAND & HAWKINS 1980). Each group of authors carried out the experiments in different modification (and not always with the use of the same inhibitors). Therefore, a comparison of their data does not allow to elucidate the interspecific characters in cell division controls. However, it is important to note that, according to the works mentioned above, various inhibitors, even at low concentrations, penetrate easily into *Volvox* cells and embryos.

Our analysis of the effects of metabolic inhibitors on the process of cleavage in *V. aureus*, *V. carteri f. nagariensis* and *V. tertius* (DESNITSKI 1985 b, 1986, 1987, 1990) demonstrates that every species is characterized by some peculiarities of cell reproduction during the asexual development. Aminopterin (the inhibitor of DNA precursor synthesis), actinomycin D (the inhibitor of RNA synthesis), streptomycin and cycloheximide (the inhibitors of protein synthesis on 70 S and 80 S ribosomes respectively) have been used in these investigations.

Table 1. The effect of inhibitors on the process of embryonic cleavage in various species of *Volvox* expressed as the mean number of cell divisions completed after placing in a solution of an inhibitor.

Inhibitor	Concentration ($\mu\text{g/ml}$)	<i>V. aureus</i>		<i>V. carteri f.</i> <i>nagariensis</i>		<i>V. tertius</i>	
Aminopterin	5	1.14	1.02	no inhibition		no inhibition	
	10	0.45	0.92	no inhibition		2.43	2.77
	25	0.24	0.42	no inhibition		2.07	2.66
Actinomycin D	5	0.67	0.66	≥ 4.79	≥ 5.35	≥ 5.00	≥ 5.16
	20	0.60	0.44	0.32	0.55	0.71	0.74
Cycloheximide	0.1	0.35	0.53	≥ 4.99	≥ 5.12	4.08	≥ 4.54
	0.4	0.28	0.23	1.26	0.79	0.75	0.60
	1.0	0.20	0.20	0.47	0.12	0.42	0.28
Streptomycin	25	2.24	2.05	not tested		no inhibition	
	100	1.06	0.67	≥ 6.15	≥ 5.86	≥ 5.97	≥ 5.63
	200	not tested		4.94	4.70	3.78	4.23
	500	not tested		0.70	0.92	1.44	1.06

The data are given in Table 1. It has been shown that cell divisions in the embryos of *V. aureus* are very sensitive to the action of all inhibitors, whereas the embryonic cleavage in *V. carteri f. nagariensis* is much more resistant. The process of divisions in *V. tertius* is also characterized by a significant resistance to the action of actinomycin D, streptomycin and cycloheximide, but it is not as aminopterin-resistant as that in *V. carteri f. nagariensis*. It should be noted that the data showing a high resistance of *V. carteri* cleavage to aminopterin are in accord with WEINHEIMER's (1983) work in which 5-fluoroacil, the other inhibitor of DNA precursor synthesis, was tested using the embryos of the same species. Thus it may be supposed that the 2-celled embryo of *V. carteri* is already provided with a large amount of the precursors for DNA synthesis. These endogenous pools of deoxynucleotides seem to support the rapid rate of cell divisions. It would be similar to the data on cell division controls in early embryos of amphibians (LANDSTRÖM et al. 1975) and sea urchins (NIKURA et al. 1984). On the contrary, in *V. tertius* and especially in *V. aureus*, the pools of DNA precursors in the early embryos appear to be comparatively small. Therefore, an important role for cell division controls in these two species plays the formation of deoxynucleotide pools throughout the process of embryonic cleavage.

It is appropriate to summarize our work with using metabolic inhibitors. The extent of sensitivity (or resistance) of cleavage process in various species of *Volvox* to the inhibitors of

transcription and translation seems to depend on the presence or absence of cellular growth during the intervals between consecutive embryonic divisions. However, the high resistance to the inhibitor of DNA precursor synthesis correlates with a rapid rate of the embryonic cleavage, but not with the lack of cellular growth.

5. Mechanisms of the evolutionary changes of the asexual development in the genus *Volvox*

In the protistological literature (see: SLEIGH 1989), the term "palintomy" is used to designate the process during which a giant parental cell undergoes the rapid sequence of repeated divisions without growth to produce numerous small cells. In the genus *Volvox* the palintomy takes place in *V. carteri*, the asexual development of which was considered above, as well as in *V. pocockiae* (STARR 1970a), *V. obversus* (KARN et al. 1974), *V. gigas* and *V. powersii* (VANDE BERG & STARR 1971). These species are characterized not only by the large gonidia, dividing with no growth, but also by the rapid rate of embryonic cleavage. On the other hand, it is evident that in *V. aureus* and in the other species with small gonidia there is no palintomy during the asexual life cycle.

What are the evolutionary relationships between the palintomic (e.g., *V. carteri*) and the non-palintomic (e.g., *V. aureus*) types of the asexual life cycle in *Volvox*? To answer this question, a number of facts should be taken into account. First, the vast data on the asexual reproduction in the unicell *Chlamydomonas reinhardtii* (LIEN & KNUTSEN 1979; SPUDICH & SAGER 1980; MCATEER et al. 1985) and the colonial *Eudorina elegans* (LUNTZ 1968; LEE & KEMP 1975) demonstrate that both are the palintomic species. Moreover, according to the light/dark control of cell divisions as well as according to macromolecular synthesis variations during development, their asexual life cycles are similar to that of *V. carteri* (but differ from the asexual cycle of *V. aureus*). Secondly, *V. powersii* and *V. gigas*, the most primitive species of *Volvox*, are also characterized by the palintomy. Thus it might be supposed that the *V. aureus* type of development is more advanced than that of *V. carteri*. However, one cannot forget that these two species belong to different taxonomic sections of the genus *Volvox*. As a matter of fact, the evolutionary relationships between Janetosphaera and Merrillosphaera sections are obscure. Nevertheless, *V. pocockiae* is characterized by the *V. carteri* type of asexual development, though *V. pocockiae* and *V. aureus* belong to the same section, Janetosphaera. Therefore, there are certain reasons to suppose that the palintomy is reduced in *V. aureus*.

On the other hand, our investigation of the asexual life cycle of *V. tertius* shows a reduction of the palintomy within the section Merrillosphaera as well. This event seems to occur irrespective of the evolutionary changes in the section Janetosphaera. In *V. tertius*, however, only some palintomic traits are reduced: some features of its asexual development are similar to the development of *V. aureus*, but the others are similar to that in *V. carteri*. The analysis of the light/dark control of cell divisions in *V. aureus*, *V. carteri* f. *nagariensis* and *V. tertius* as well as the experiments with the inhibitors have enabled us to elucidate partly the mechanisms of the evolution of asexual ontogenesis in *Volvox*. Thus the initial step of the palintomic reduction (seen in *V. tertius*) is probably connected with 1. a change in the light/dark control of the process of cleavage, 2. a slowdown of cell divisions and 3. changes in the metabolism of DNA precursors. However, there are no accompanying changes in the dynamics of RNA and protein syntheses during the asexual life cycle. In this respect *V. tertius* appears to be similar to the palintomic species, *V. carteri*. It may be due to the lack of cellular growth during the embryonic cleavage in all members of the section Merrillosphaera.

To investigate further the evolution of ontogenesis in *Volvox*, it would be helpful to unite the comparative approach, which was used in our work, with the genetic and molecular

approaches used by other authors (HUSKEY & GRIFFIN 1979; KIRK 1988; RAUSCH et al. 1989). First, it would be of interest to search for the mutations of *V. carteri* f. *nagariensis* with slow divisions of the embryonic cells. Secondly, it is very important to elucidate the evolutionary relationships among various species and sections of *Volvox*, using the method of ribosomal RNA sequence. In particular, it would uphold or refute our assumption that a slow rate of the embryonic cleavage may serve as a criterion for the advanced phylogenetic position of a species within the genus or a section of *Volvox*.

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