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BUILDING A MULTICELLULAR ORGANISM

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■ **Abstract** Multicellular organisms appear to have arisen from unicells numerous times. Multicellular cyanobacteria arose early in the history of life on Earth. Multicellular forms have since arisen independently in each of the kingdoms and several times in some phyla. If the step from unicellular to multicellular life was taken early and frequently, the selective advantage of multicellularity may be large. By comparing the properties of a multicellular organism with those of its putative unicellular ancestor, it may be possible to identify the selective force(s). The independent instances of multicellularity reviewed indicate that advantages in feeding and in dispersion are common. The capacity for signaling between cells accompanies the evolution of multicellularity with cell differentiation.

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INTRODUCTION

When we look at life around us, multicellular organisms—plants, animals, and colonial microorganisms—meet our eyes. It is generally believed that single cells were first to evolve, but the oldest fossils of ancient life, 3500 million years

(Myr) of age, show multicellular cyanobacterial filaments (44). The 2-methylbacteriohopanepolyols that characterize the cyanobacteria are abundant in organic rich sediments as old as 2500 Myr (48). The transition to multicellularity is unusual among the major transitions in evolution (33) in that it has occurred numerous times. Novel multicellular forms are still being discovered (37). By a multicellular organism, we understand one in which the activities of the individual cells are coordinated and the cells themselves are either in contact or close enough to interact strongly. According to this definition, a bacterial colony is not a multicellular organism, even though it may show patterned growth, because it apparently lacks overall coordination of function.

The multicellular condition has independently arisen in each of the organic kingdoms, and in some phyla several times in the course of their evolution. For example, the sponges are believed to have arisen from the choanoflagellates separately from all other animals; the seed plants, the fungi, the brown algae, and the red algae all gained their multicellularity in separate events (53). The same is true among microorganisms: After the cyanobacteria, the cellular slime molds, the myxomycetes, and the myxobacteria independently adopted multicellularity (5). These transitions can be viewed as repetitions of an experiment of nature that tests the advantages of multicellular life. The repetition challenges biologists to identify their similarities. An aim of this review is to compare, for a sample of three independent steps to multicellularity, the growth and fitness of the multicellular organism with those of a possible unicellular ancestor.

Life's unicellular origin implies that the fundamental genetic and epigenetic systems of all animals and plants were originally devised for those single cells (5). If so, how were those systems enhanced to manage a multicellular life cycle? A second aim of this review is to look for enhancements by comparing the regulatory circuits of single and multicellular forms. Were any new modes of epigenetic control needed? Practically complete parts lists are now available for a multicellular cyanobacterium (*Nostoc*), for *Caenorhabditis elegans*, for *Drosophila melanogaster*, for *Homo sapiens*, and soon for mice and zebrafish. Already, specific enhancements have been proposed for the signaling in metazoa (34, 38, 47). Scrutiny of the completed multicellular genomes may reveal traces of their origins. The challenge is to read those traces.

CYANOBACTERIA

Filamentous cyanobacteria are the Earth's oldest known multicellular organisms (44). Fossilized remains discovered in the 3465-Myr Warrawoona sedimentary rocks of northwestern Australia record organisms that may have lived when the Earth was only 1 billion years old (Earth's estimated creation at 4700 mya). The fossils show unsheathed filaments with terminal and medial cell shapes that resemble modern species of Oscillatoria, such as *Oscillatoria grunowiana*, *O. chalybea*, and *O. antillarum* (44). Whether the fossilized organisms carried out oxygenic

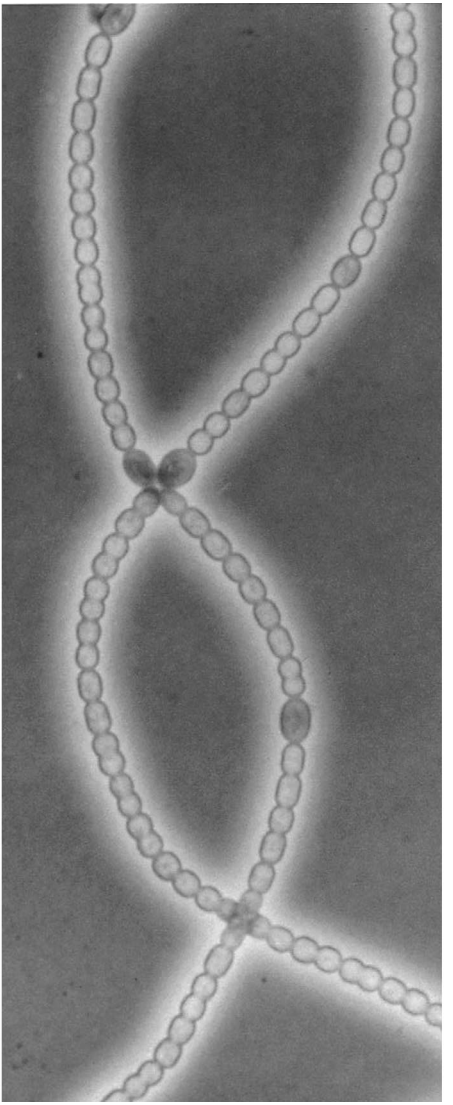
photosynthesis with chlorophyll a and photosystems I and II, like their modern counterparts, or were anaerobic has not been settled (45). However, the 2-methylbacteriohopanepolyols that characterize modern aerobic cyanobacteria are abundant in organic rich sediments as old as 2500 Myr (48). Moreover, the necessary CO_2, H_2O, and light would have been available when the fossils were alive, and the isotopic carbon ratios contained within the organic matter of the fossil (kerogen) are compatible with oxygenic photosynthesis by these organisms (16). Assuming that life began with single cells, the antiquity of these multicellular filaments, as well as their morphological diversity, imply that multicellularity was advantageous on the Archaen Earth.

In addition to photosynthesis, many extant species of cyanobacteria fix atmospheric dinitrogen. However, because nitrogenase, the fixation catalyst, is very oxygen-sensitive, cyanobacteria arrange to separate nitrogen fixation from photosynthesis either temporally or spatially. In some genera, like *Nostoc*, aerobic nitrogen fixation is confined to differentiated cells called heterocysts. The ability to form heterocysts probably evolved over 2 billion years ago, but following the first filamentous forms (13). O_2 sensitivity of nitrogenase may have been a factor in their selection, but the atmospheric O_2 concentration at the time of their appearance is a much discussed matter (45). The data, despite their uncertainties, suggest that a major selective force in the evolution of the multicellular cyanobacteria was access to a more efficient and a more universal source of environmental nutrition. They became autotrophs capable of living on substances of high abundance at the surface of the earth: CO_2, H_2O, N_2, light, and inorganic ions.

Figure 1*A* shows a long chain of *Nostoc* vegetative cells in which several heterocysts are visible as larger darker cells. Figure 1*B* is an electron micrograph of a section through three vegetative cells and a thick-walled heterocyst at the right. Glutamine carries fixed nitrogen from the heterocyst to neighboring vegetative cells in the filament, whereas the photosynthetic vegetative cells supply organic carbon and the reducing power (NADPH) necessary for N_2-fixation in the heterocysts (1, 49). Heterocysts lack the oxygen-evolving photosystem II activity. They also have a laminated glycolipid layer outside the cell envelope that reduces the diffusion of gases (52). Heterocysts are thus able to maintain a relatively anoxic microenvironment in a filament that is oxygen rich. Heterocysts also lack ribulose bisphosphate carboxylase and so do not fix CO_2. A *Nostoc* filament thus lives as a nitrogen-fixing photoautotroph by virtue of its multicellular condition.

Recently, the Joint Genome Institute released a draft of the 9.76-Mb *Nostoc punctiforme* genome: 7432 ORFs were detected (18). The ensemble of the putative ORF functions sketches a rough metabolic and regulatory picture of *Nostoc*. For comparison, the full sequence of the unicellular, phototrophic cyanobacterium, *Synechocystis*, of 3.6 Mb has also recently been released (11).

Synechocystis, shown in Figure 2, can also use NH_4^+ and NO_3^- as nitrogen sources, but cannot fix N_2. A genomic relationship to the multicellular *Nostoc* is revealed by the fact that 80% of the *Synechocystis* genes are significantly similar,



(A)

(B)

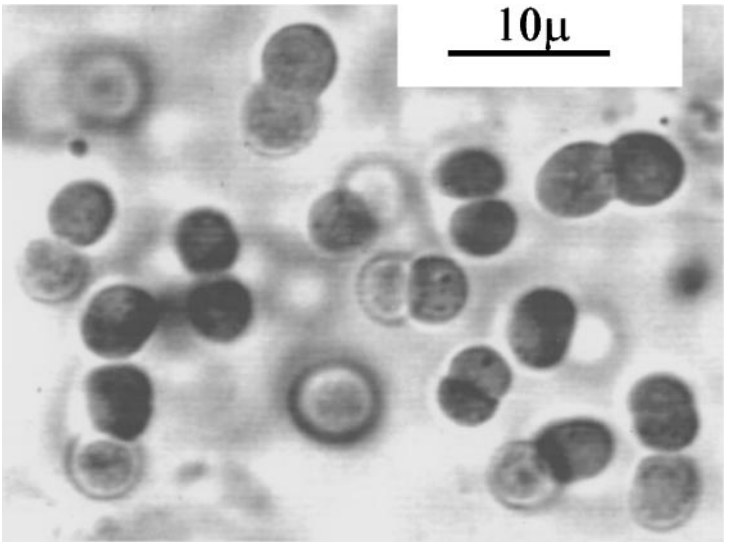


Figure 2 Culture of *Synechocystis* sp. Several cells are dividing. Photo courtesy of Cyanosite. Web address <www.cyanosite.bio.purdue.edu/images/images.html>

with BLAST expectation values less than e^{-5}, to one and often to several *Nostoc* genes that are also assigned the same function (11). Both 16S rRNA sequences of *Synechocystis* are 89% identical in BLAST alignments with the 16S rRNA sequences of *Nostoc*, and so it is possible but uncertain whether the latter arose from the former. Nevertheless, comparison of the parts list can suggest which functions are required to manage multicellularity. It is generally believed that core metabolic, transcriptional, translational, and replicative functions evolved for unicells (5). A filamentous form may then have arisen by failure of two daughter cells to separate after division. If the filament had some advantage over the unicell, it required preservation, and various regulatory devices would have evolved to manage what had become a true multicellular organism.

What might those devices be? There are 7400 protein genes in the sequenced strain *Nostoc* PCC 73102, and 3200 protein genes in *Synechocystis* PCC 6803, leaving 4200 genes, some of which might encode new devices. Functional analysis of those 4200 genes awaits a final annotation, but differences between the *Nostoc*

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Figure 1 *A. Nostoc punctiforme*, light photomicrograph. *B.* is an electron micrograph of a section through three vegetative cells and a thick-walled heterocyst at the right. Note the connection between the heterocyst and the adjacent vegetative cell. Both photographs courtesy Dr John C. Meeks, University of California, Davis.

TABLE 1 Signaling proteins

Function	***Synechocystis* 6803**	***Nostoc* 73102**
Total	3200	7400
Histidine kinase	20	146
Response regulator, receiver	48	168
Ser/Thr protein kinase	8	51
HTH regulator	1	100
Sigma-70 forms		13

draft and the final annotation of *Synechocystis* are already apparent in the numbers and proportions of genes that encode components of signal transduction pathways. These are components of signal production and signal reception circuits. There are histidine protein kinases, often associated with sensory function, reception domains of response regulators, serine/threonine protein kinases, helix-turn-helix proteins, and alternative sigma factors. Table 1 offers a comparison of the numbers and proportions of these classes of proteins in the two genomes. Differences between the two are apparent in the numbers and in the proportions of all genes.

Evidence for added regulatory devices to manage the multicellular state also follows from genetic studies on the differentiation of heterocysts. *Anabaena* (also known as *Nostoc* PCC 7120) forms chains of photosynthesizing vegetative cells, punctuated with an occasional heterocyst. The sequenced multicellular *Nostoc* PCC 73102 shares 73% of its genes with *Anabaena*. If *Anabaena* is grown in a medium that provides ample fixed nitrogen as NH_4^+, nitrogen fixation is not required and no heterocysts are formed, only vegetative cells. But when cells that have been grown with ample NH_4^+ are washed, then resuspended in medium free of fixed nitrogen, they develop heterocysts during the next 24 h. Since the vegetative generation time under these conditions is also about 24 h, heterocyst differentiation can be thought of as growing a new cell with a different wall and a somewhat different set of enzymes. The RNA hybridization data suggest as many as 1000 protein differences between vegetative cells and heterocysts (7).

When nitrogen fixation is needed, about 1 cell in 10 becomes a heterocyst. N_2 fixation and respiration in the heterocyst require a supply of reductant and of carbon from the adjacent, photosynthesizing, vegetative cells. Reductant and carbon are provided in the form of maltose, sucrose, or other disaccharides. In return, the heterocyst releases fixed nitrogen in the form of glutamine to its vegetative neighbors.

The intercellular exchange of metabolites illustrates the metabolic interdependence of vegetative cells and heterocysts. Wilcox (54) noted that these cells are also developmentally interdependent in that the heterocysts differentiate at fairly regular spatial intervals, which is evident in Figure 1*A*. Chain-breaking experiments show that many more cells have the potential to become heterocysts than

normally do so (54). Because all the cells in a chain have received the same environmental cue—the paucity of ammonium ion, some kind of cell interaction has been sought to explain the regular spatial pattern of heterocysts. If all cells in a chain of vegetative cells grow then divide, the chain elongates. To maintain a fixed ratio of heterocysts to vegetative cells, new heterocysts would need to differentiate in proportion to the new vegetative cells that form. Moreover, a regulatory system that appropriately selects particular vegetative cells to become heterocysts would seem to be necessary so that fixed nitrogen (glutamine) will be available to all the vegetative cells. Since heterocysts and vegetative cells differ in their levels of hundreds of proteins, the process needs coordination. It is hard to escape the inference that a cell interaction triggers one daughter cell to become a proheterocyst and its sister to remain a vegetative cell.

Each new heterocyst forms very near the center of a segment of vegetative cells after their number has doubled. This location maintains a stable ratio and spatial distribution of the two cell types, presumably optimized for exchange of fixed carbon and fixed nitrogen between vegetative cells and heterocysts. But what are the cell interactions and how do they generate the pattern? A promise of answers to this question comes with the discovery of several genes that alter the heterocyst pattern. Major candidates are *hetR*, *patA*, and *patS*. Mutations in *hetR* eliminate heterocysts, whereas multiple copies of *hetR* stimulate the formation of heterocysts in the presence of combined nitrogen, and the formation of clusters of heterocysts in its absence (6). *patA* mutants are unable to develop interstitial heterocysts; they form terminal heterocysts only (30). A *patA* mutation suppresses the multiple heterocyst phenotype of *hetR* mutants. Overexpression of *patS* blocks heterocyst differentiation, while a *patS* null mutant has an increased frequency of heterocysts—clustered abnormally along the chain of cells (56). Since *hetR* and *patA* are increasingly expressed after nitrogen step-down in vegetative cells, this set of properties suggests that *hetR* and *patA* combine to induce heterocyst differentiation, while *patS* encodes an inhibitor of that process (2, 55).

Moreover, *patS* is expressed in proheterocysts but not in vegetative cells. The *patS* gene can encode a peptide of 17 amino acids, and a synthetic peptide corresponding to its C-terminal pentapeptide has the capacity to inhibit heterocyst development. Yoon & Golden (56) propose that heterocysts synthesize and secrete a *patS* peptide that prevents neighboring vegetative cells from becoming heterocysts.

MYXOBACTERIA

Some organisms become multicellular by aggregation rather than by growth and cell division. Myxobacterial cells aggregate to build sometimes quite elaborate fruiting bodies within which they sporulate. The life cycle of these prokaryotes is surprisingly similar to development of the (eukaryotic) cellular slime molds, such as *Dictyostelium* (4, 20). All myxobacteria form fruiting bodies and no unicellular

species have been found. Perhaps the multicellular myxobacteria replaced their unicellular progenitors from a common ecologic niche.

Myxobacteria lie on the boundary between uni- and multicellular organisms. Although they grow and divide as proper Gram-negative bacteria, they constitute a primitive multicellular organism whose cells feed socially as multicellular swarm units. They eat particulate organic matter in the soil using a variety of secreted hydrolytic enzymes: lysozymes, proteases, and cellulases. They feed like packs of microbial wolves. Their cooperation is demonstrated when they are provided with a nutrient polymer like casein. Growth on casein is faster at higher cell density (42). When fed casein digested with the proteases in a culture supernatant, then low-density cultures grow at the high-density rate.

They also build multicellular fruiting bodies, five species of which are shown in Figure 3. When they have exhausted their food, 100,000 cells build these compact and symmetrical structures, often with stalks, branches, and multiple cysts evident in the Figure.

According to the 16S ribosomal RNA sequence of these organisms and to other chemotypic markers, the various species in this photo are phylogenetically related. Molecular genetic studies show that both *Myxococcus* and *Stigmatella* have circular genomes of about 9.5 Mb (8, 35). Immediately the question arises: Why do myxobacteria have more DNA than *Bacillus subtilis*, for example, which efficiently makes notoriously good spores (46)? Each myxobacterial cyst is a package of spores. As soon as food becomes available, the spores germinate and organize a feeding swarm that is thus able to grow. A selective advantage for their multicellular state is their cooperative feeding. Myxobacteria feed on particulate organic matter in the soil, which they digest with a battery of secreted hydrolytic enzymes, proteases, lysozymes, nucleases, and in some cases cellulase (43).

The selective advantage of multicellular fruiting bodies is probably twofold. One advantage is that immediately after the spores germinate from the same fruiting body, they can cooperate for efficient feeding. A second advantage relates to the macroscopic dimension of a fruiting body, which effectively gives legs to the spores. The fruiting body package of 0.2 mm, or more, is large enough to adhere to an animal that happens to brush by it in the soil and then to be carried away by that animal. Fruiting bodies have been seen on the backs of mites (40). A passing insect or worm in the soil is likely to be searching for food. The animal carries, then deposits, that fruiting body on its food, providing food for the myxobacteria as well. While sporulation is a great strategy for survival when food runs out, multicellular sporulation adds the possibility of transport to a new place where starving cells can refresh themselves and grow.

A spore-transport/cooperative-feeding advantage is suggested by the fact that only the cells inside the rounded masses of a *Stigmatella* or *Chondromyces* fruiting body, or the spherical mound of *Myxococcus xanthus* or *M. stipitatis* differentiate into spores (19). The point is that the masses are more likely to be plucked for purely mechanical reasons (see Figure 3). Spores outside the masses or adhering

directly to the substrate are less likely to be picked up by a passing animal. Though plausible, this suggestion needs to be tested.

A- and C-Signaling

How spores become localized is explained by cell-to-cell signaling. Sporulation is the final step in a developmental program that starts from a disorganized biofilm of growing cells (Figure 4, *top*). In 4 h, irregular aggregates of ~1000 cells appear. Then, many cells stream into these foci from all directions so that by 24 h a hemispherical mound of 10^5 cells has been created. When such a mound is cracked open it is seen to be densely packed with spores. At least two extracellular signals are necessary for making spores. Signaling to coordinate sporulation might require an even larger set of genes than the structural changes that differentiate a spore. For this purpose, myxobacteria may have many genes to make and to export the signals, as well as to receive and to interpret them accurately.

Isolated and purified from medium conditioned by developing cells, A-factor is a set of 6 particular amino acids: trp, pro, phe, tyr, leu, and ile. A-factor, like the homoserine-lactones in enteric bacteria, is a quorum sensor. Each cell produces a fixed amount of A-factor. However, only cells whose nutritional state indicates severe starvation release A-factor. Moreover, there is a response threshold for the A-signal. Therefore, a certain minimum number of cells must agree that the population should commit itself to fruiting body development even before aggregation starts. Note that the signaling concentration of A-factor amino acids is about tenfold lower than the concentration necessary to support growth (29).

Purified C-factor, by contrast to A-factor, is an approximately 20-kDa protein. It has a hydrophobic N-terminal that keeps it associated with the cell surface. The properties of C-factor (water insoluble and cell surface bound) are appropriate to the disposition and density of cells at the time of C-signaling. In the beginning when the cells are at relatively low density, A-factor amino acids must diffuse from cell to cell. Later, when the cells have aggregated to a 1000-fold higher density within a nascent fruiting body, C-signaling starts. C-factor is bound to the cell surface, and C-signaling requires end-end cell contact for signal transmission (22).

Mutants defective in A-signaling (*asg* mutants) fail to assemble fruiting bodies and fail to sporulate. Mutants defective in C-signaling (*csg* mutants) are blocked with only the early asymmetric aggregates having formed. They fail to assemble hemispherical mounds and they fail to sporulate.

Response to Signals

Starvation, the A-signal, and the C-signal order the time of gene expression for the program of fruiting body development. This diagram (Figure 5), invented by Kroos (21), shows how developmentally regulated genes are induced at appropriately different points in time as a consequence of their signal dependence. The signals act directly or indirectly as transcription factors. Starvation, i.e., the lack of any

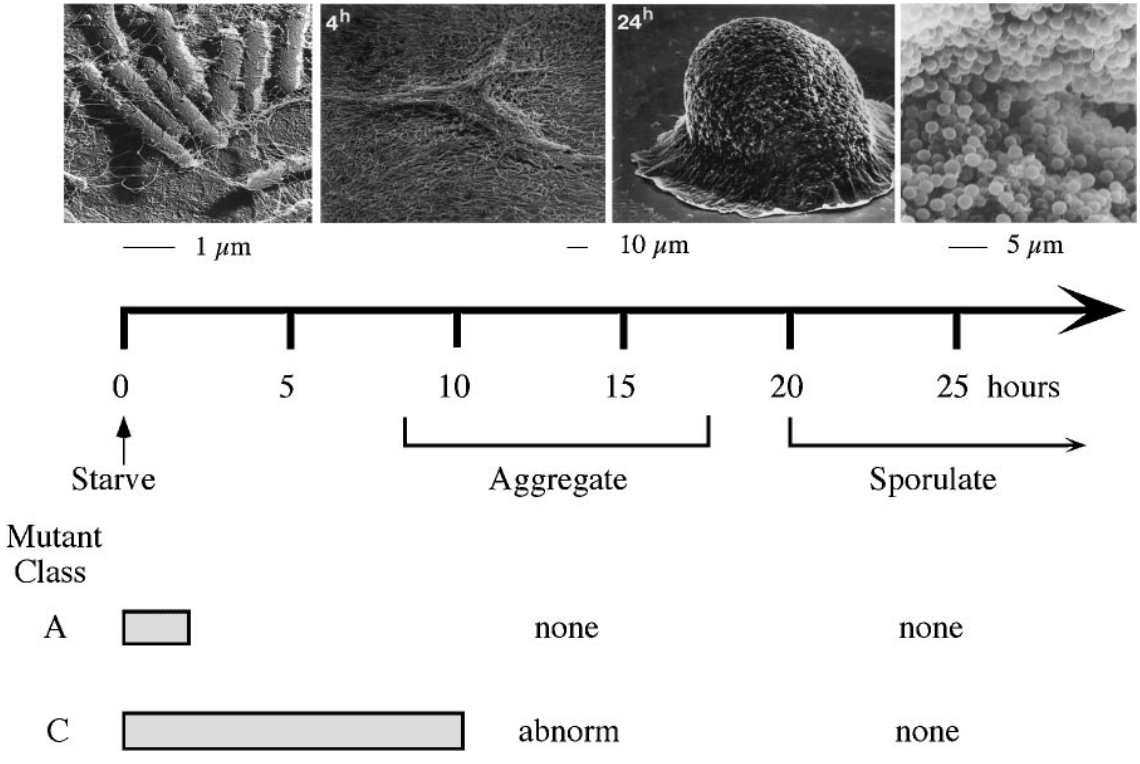

Figure 4 Fruiting body development in *Myxococcus xanthus* depends on the A- and C-signals. *Top*: Scanning electron micrographs of fruiting body development viewed at the start, at 4 h, at 24 h, and after cracking the fruiting body open. *Below the time scale* are shown the aggregation and sporulation processes. A-signaling mutants express a few early developmentally regulated genes, but neither aggregate nor sporulate. C-signaling mutants express more developmentally regulated genes, aggregate at the 4-h stage, but do not sporulate.

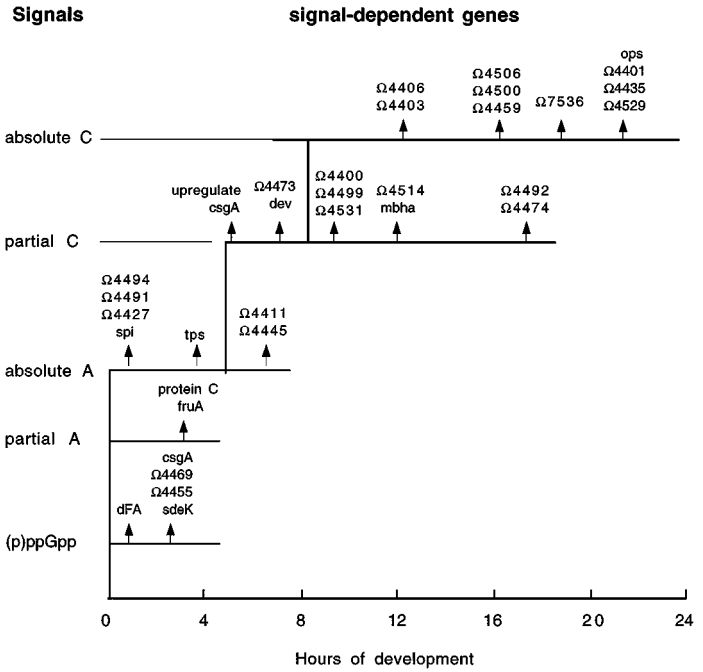

Figure 5 Expression of signal dependent genes. Genes have lower case names or Ω numbers. The arrow beneath a gene indicates the time at which it begins to be expressed. The signals are listed at the left in the order, from bottom to top, in which they are produced during development. The horizontal lines at the level of each signal divide the genes into those above the line, which are expressed with each signal and those below the line, which are independent of the signal. Absolute means that there is no expression in the absence of the signal; partial means that there is some expression in the absence of the signal but much more when the signal is present (26, 28).

one or more of the amino-acylated tRNAs induces a stringent response and the synthesis of (p)ppGpp by *Myxococcus* (14). This highly phosphorylated guanosine nucleotide apparently initiates the developmental program. Some genes, such as *sdeK*, require only (p)ppGpp for expression and they are expressed early, before 2 h of development. The next set requires A-factor, which is released around 2 h of development in addition to (p)ppGpp (29). The various developmental gene products are indicated by name in the diagram of Figure 5, and promoters that fire at the time shown on the horizontal axis by an omega 4-digit number. Genes that are indicated as "partial A" on the vertical axis begin to be expressed before A-factor is produced, but their expression increases twofold or more when A-factor is released at 2 h (28). Others, denoted "absolute A," are totally dependent on A-factor: no signal, no expression.

Consequently, A-factor-deficient mutants arrest development having expressed only those genes below the "absolute A" line shown on Figure 5. No gene above that line is expressed. As mentioned above, A-signal helps to evaluate starvation. The proteins charted in this diagram, and other proteins (since the screen for such genes is not yet saturated) are synthesized during assembly of the fruiting body and differentiation of spores. To be able to synthesize these developmental proteins, aggregation and sporulation must be initiated while the cells still retain some capacity for protein synthesis. In other words, a starving population must have anticipated the future absence of nutrient when it started to build a fruiting body. Individual cells register their vote in favor of building a fruiting body by releasing the set of A-factor amino acids, once they have made a grim evaluation of the nutrient available.

The C-signal is a morphogen that organizes the fruiting body and limits sporulation to its interior. C-factor-deficient mutants are blocked below the "absolute C" line in Figure 5, expressing the early A-signal-dependent, but C-signal-independent genes. Some essential sporulation genes are absolutely C-signal dependent. The spatial confinement of sporulation to the interior of the fruiting body is shown with the aid of a transcriptional fusion between the green fluorescent protein (GFP) and a sporulation promoter. The fusion strain was induced to develop fruiting bodies. A fruiting body in which sporulation has begun is shown photographed in visible light in the left panel of Figure 6.

Localized Gene Expression

The fruiting body is dense because the cells within are close packed. The fruiting body is surrounded by cells at much lower density. The surrounding rod-shaped cells are organized into many raft-like clusters. Even as many of the fruiting body cells are differentiating into spherical spores, none of the peripheral cells are doing so; all retain their rod shape. The very same microscopic field under UV illumination to excite the GFP is shown in the right panel of Figure 6. Only the fruiting body fluoresces; none of the peripheral cells fluoresce. All the cells in this experiment, whether inside the fruiting body or out, carry the same GFP transcriptional fusion. Why might an apparatus that localizes sporulation have been selected in evolution? The fruiting body/sporulation process is induced by starvation; by the time (24 h) that sporulation commences, the amino acid reserves are in short supply, and yet new proteins must be made. The very limited capacity of the cells for protein synthesis at this point requires it to be used where it counts most. Perhaps the rigors of starvation explain why sporulation is spatially restricted: Spores at the top of the fruiting body are in the best position to be picked up and carried to a new place.

Natural selection for social behavior has been explored in long-term population experiments. Twelve parallel suspension cultures were propagated continuously for many generations under conditions that required neither cooperative feeding nor multicellular fruiting body development. Each culture was repeatedly sampled

on the way to 1000 generations. A majority of the cultures contained mutants that had lost fruiting body development and sporulation efficiency, suggesting that these social behaviors are detrimental to fitness under such asocial growth conditions (50). Maynard Smith (33) pointed out that when the multicellular stage of myxobacteria is formed anew in each cycle by aggregation, there is a danger that selfish mutant cells will disrupt multicellular organization. Indeed, Velicer et al. showed that some developmental mutants are cheaters (51). When such mutants were mixed in low proportion with wild-type (WT) cells, and the mixture induced to develop fruiting bodies, the mutants represented a disproportionately large number of the spores. Cheaters had never been reported to accumulate in laboratory cultures that arise from the spores of fruiting bodies. However, these anecdotes need to be replaced by systematic observations. Cheaters may be infrequent in natural populations because Velicer and colleagues also observed that fruiting bodies with many cheaters contained fewer spores than pure WT cultures (51).

Cooperative feeding is not always linked to the formation of fruiting bodies. Many gliding bacteria in the soil, such as the *Cytophagas* and the *Flexibacters*, feed with extracellular enzymes but do not build fruiting bodies. Although some, such as *Sporocytophaga*, do sporulate, they do so as single cells. Myxobacteria are found in all climate zones, vegetation belts, and altitudes (41). They compete effectively with the cellular slime molds, which inhabit the same ecological niche in soil. Well-cultivated and aerated soils often contain 10^6 myxobacteria per gram. Two forces appear to have combined in selecting the myxobacterial grade of multicellularity: more efficient feeding and more efficient dispersal.

To what extent does an infrastructure required for social behavior explain why *M. xanthus* has a genome of 9.5 megabase pairs, larger than almost all other bacteria? The gene density, average gene size, and absence of repeat sequences are comparable to *Escherichia coli* K12 in the 1% of the *M. xanthus* genome whose sequence is published. They are *E. coli*-like overall, according to the Monsanto/Cereon sequencing group who have completed a 4X sequencing of the *M. xanthus* genome. Annotation is under way that will give a draft inventory of gene functions. That inventory will test whether cell-cell signaling plays a significant role in determining its genome size.

VOLVOX

This photosynthetic green alga (Figure 7) lives in sunlit, standing waters. Many plants compete for light and nutrients in such places, including unicellular and various grades of multicellullar green algae. The family *Volvocaceae* contains about 40 multicellular species, all closely related to one of the unicellular members of that family, like *Chlamydomonas reinhardtii*, which is also in Figure 7. The relationships of their cytology, physiology, and phylogenetics support the hypothesis that *Volvox* evolved from *Chlamydomonas*. The richness of the cell biological and physiological data for the photosynthetic green algae allows comprehensive comparisons to be made between *Chlamydomonas*, *Volvox*, and their relatives.

Phylogeny

Fossils of unicellular algae have been reported from the Precambrian (9). Although the identity of these species has yet to be established, the fossils imply that green algae had arisen by that time. Based on sequence comparisons of the complete small ribosomal RNA gene (39), *Volvox carteri* is as closely related to *Chlamydomonas reinhardtii* as the two grasses, corn (*Zea mays*) and rice (*Oryza sativa*), are to each other. Kirk (23) estimates that corn and rice shared their last common ancestor about 50 mya, providing an estimate for the divergence between *Chlamydomonas* and *Volvox*. Extensive comparisons of the two internal transcribed spacer regions of the nuclear rDNA confirm the notion that *C. reinhardtii*, among a series of other green unicellular members of the *Volvocaceae*, is the most similar to *V. carteri* (10, 32).

Historically, the genus *Volvox* has been defined with morphologic, not phylogenetic, criteria. Later ribosomal RNA comparisons as well as the sequences of the genes for the ATP synthase beta-subunit and the large subunit of the CO_2-fixing enzyme, ribulose 1,5-bisphosphate carboxylase (36), indicate that the morphologic genus *Volvox* is probably polyphyletic (10, 36). Nevertheless, according to these and other molecular comparisons, the various *Volvox* clades are all related to *C. reinhardtii*. Thus, *Chlamydomonas*, or its progenitor, may have gained a morphologically similar grade of multicellularity several times in the past 50 million years.

A variety of multicellular states are represented by *Volvox*, and by *Gonium*, *Pandorina*, *Eudorina*, and *Pleodorina*, which differ in the number of *Chlamydomonas*-like cells they contain. This variety may exist because, being photosynthetic, sunlight provides the bulk of their energy, and each cell in the community absorbs light; their evolution need not have been constrained by the need for inventing a communal feeding apparatus, which was needed for the sponges (5). The wide range of multicellular forms represented by *Volvox*, *Gonium*, *Pandorina*, *Eudorina*, and *Pleodorina* implies either that the multicellular transition occurs easily, or that the multicellular state has a substantial selective advantage. Ease is suggested by the tendency of sister *Chlamydomonas* cells to cohere to each other after division, surrounding themselves with a mucilaginous polysaccharide, and spontaneously generating a multicellular array of nonmotile cells. This tendency is common enough to be named a palmelloid stage. Similarly, *Gonium* forms disks of 4, 8, 16, or 32 *Chlamydomonas*-like cells; the flagella of each cell beat independently; each cell divides independently and is capable of regeneration. However, the evidence to be discussed suggests that such arrays are not functionally integrated in the way that *Volvox* is. This crucial difference motivates the distinction between colonial and multicellular organisms made for this review.

Modern *Chlamydomonas* lives with modern *Volvox*. Mixed populations of green algae are found in small pools, puddles, and temperate lakes. These waters tend to be unmixed and turbid. They are typically nutrient rich, providing sources of fixed nitrogen, phosphorus, and sulfur (23). Cohabitation with *Chlamydomonas* suggests

that any multicellular advantage in growth has not caused *Volvox* to replace the unicells with which they compete. This may result from the facts that illumination and nutrient availability tend to change with the season, and puddles tend to dry up or freeze. Cyclic changes in temperature and light may have prevented replacement or even the establishment of an equilibrium population. In lakes and ponds, *Volvox*, *Pandorina*, and *Eudorina* fluorish only briefly each year (23). With such seasonal changes, motility and phototaxis are essential for photosynthetic organisms.

Advantages of Multicellularity

Certain advantages accrue simply from a larger size. The larger *Gonium*, *Eudorina*, and *Volvox* colonies escape from predation by filter-feeding rotifers and small crustaceans (23). Daughter colonies of *Volvox*, which would be small enough to be eaten by these animals, are kept internally, protected inside their mother colony. Another important advantage is that the larger colonies can absorb and store essential nutrients more efficiently (23). Inorganic phosphate is often a limiting nutrient for algae (3). Large multicellular algae have an advantage in phosphate uptake, storing any excess as polyphosphate in the extracellular matrix that separates the cells (25). Other nutrients may also be retained in the matrix, such as minerals, ions, and water that would help protect against desiccation. Many algae are dispersed by waterbirds (23); the larger colonies may have a better chance than unicells to be carried.

Multicellularity Exacted A Price

Volvox obtained the advantages of multicellularity after having paid a price. Insofar as that price measures the balancing advantages, that price is of interest. The vast majority of the 50,000 cells in a *Volvox* colony are specialized somatic cells that are sterile. If they are removed from the colony, they are incapable of regeneration (27). Only the germ cells of *Volvox* have offspring. By contrast, individual cells of the multicellular *Eudorina* are capable of regeneration (27). Thus the loss of reproductive potential is not inherent in this colonial form; *Eudorina* is, however, smaller and has many fewer cells than *Volvox*.

A second element is the cost of reorganizing basic cytoskeletal structures required for *Volvox* to survive. To keep the intensity of light they receive within a range favorable for photosynthesis, all swimming photosynthetic organisms strive to maintain a euphotic position in the water column. The favored intensity also must not be so high as to produce photo damage. Phototaxis, both positive and negative, is used to maintain that optimal position in the water column. However, the problem for photosynthetic flagellates is that cell division and motility are incompatible. The flagellum is constructed from microtubules and the centriole. The mitotic spindle is a mutually exclusive arrangement of the microtubules and the centriole (23a). Both *Chlamydomonas* and *Volvox* need to be motile to efficiently utilize light even as they grow and divide. Ordinarily, flagella are lost during the

mitotic cycle. Having a buoyant density greater than one, nonmotile organisms fall out of their euphotic zone.

The flagellates have variously resolved the competition for their microtubular cytoskeleton. Whereas almost all cells grow twofold before dividing once, *Chlamydomonas* and its relatives, in response to their special need for motility, regularly grow up to 32-fold, then rapidly divide (up to) five times in succession (31), apparently shortening the period of their nonmotility. Under illumination conditions resembling nature, daylight hours are devoted to growth and *Chlamydomonas reinhardtii* tends to confine division events to the night, when maintaining height is not critical. Some multicellular species use the *Chlamydomonas* solution, but not those, like Volvox, with more than 32 cells (23).

Colonies larger than 32 cells apparently need a different way to sort out the conflict between motility and cell division. The *Volvox* solution is to differentiate a subset of cells in the anterior end of the growing organism that do not divide, continue beating their flagella, and thereby provide the colony with a continuous source of photo-responsive motility. Meanwhile the rest of the cells in an immature colony divide and produce progeny. After growth is complete, the mature colony is always prepared to swim because its somatic cells no longer divide (24). The specialized germ cells are the only ones that retain the need and capacity to divide, but they are small in number. In addition to creating cells that can no longer reproduce, another cost is incurred for a cytoskeletal rearrangement that permits the cells in the mature colony to coordinate the rowing movements of all their individual flagellae.

The Cytoskeleton of All Cells in a *Volvox* Colony Must be Coordinated

Flagellar motility is necessary for *Volvox* to maintain a proper height in the water column in order to carry out photosynthesis, and this necessity appears to have played an important role in its evolution from the motile, photosynthetic *Chlamydomonas* (24). For the mature somatic cells to move the multicellular colony as a unit, they must each undergo a remarkable structural differentiation. A spherical *Volvox* colony consists of 500–60,000 individual cells, depending on the species, embedded in a common matrix. The cells are connected to each other by fine cytoplasmic bridges that remain from incomplete cell separations at cytokinesis. Those strands break down in *V. carteri* after embryogenesis, but by then the pole of each cell has become correctly oriented relative to the head of the spheroidal *Volvox* colony. Those cytoplasmic bridges had fixed the orientation of the axis of each individual cell relative to the anterior-posterior (A-P) axis of the colony producing the organized arrangement diagrammed in Figure 8.

To execute phototaxis, the colony depends on the oriented beating of all 100,000 flagella (2 flagella per cell times 50,000 cells). Both flagella on each cell must be properly oriented with respect to that cell's latitude, longitude, and the A-P axis of the spheroidal colony (Figure 8). The eyespot of each cell, which detects the incoming light, is also systematically oriented. The pair of flagella in *Chlamydomonas*

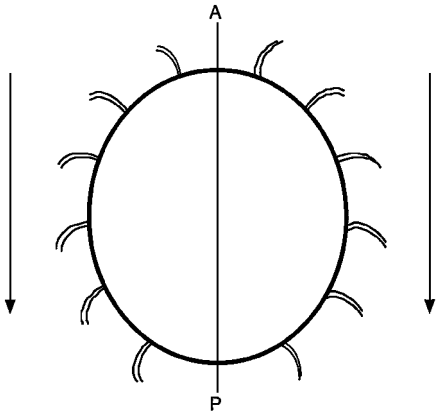

Figure 8 Orientation of cells within a *V. carteri* spheroid indicated by their pairs of flagella. A plane containing the anterior/posterior axis is illustrated. Somatic cells are oriented so that all flagella beat with their effective strokes directed from the anterior pole (*A*) toward the posterior pole of the spheroid (*P*), as indicated by the two arrows at the left and right. The posterior pole contains the reproductive cells, or gonidia, inside the spheroid. Modified after Hoops (15).

and in the immature somatic cells of *Volvox* are related to each other by 180° rotational symmetry about the cell's own axis. By means of this symmetry, a *Chlamydomonas* cell swims with a kind of breaststroke as its two flagella beat in opposite directions but in the same plane (15). By contrast, the two flagella of each mature somatic cell in a *Volvox* colony are oriented to beat in parallel planes, and in the same direction. That direction must vary systematically from cell to cell in such a way that the whole colony can progress in the direction of its (fixed) A-P axis (Figure 8) (15). As a consequence of this organized beating of flagella, the colony rotates slowly about its A-P axis as it moves anteriorly, justifying its name as the "fierce roller."

Because the direction of the effective stroke of each flagellum is fixed by the arrangement of its microtubules, maturation of *Volvox* somatic cells involves a one-time rotation of their constituent flagellar axonemes. Rotation is complete before the developing embryo breaks free of its parental spheroid. The two flagellar axonemes rotate in opposite directions, transforming the 180° rotational symmetry of *Chlamydomonas* into the requisite parallel orientation of mature *Volvox*. When the two flagella of each cell beat in the same direction, they can contribute appropriately to the movement of the entire colony. Also, the photosensitive eyespot moves in each cell from a position nearer one of the flagellar bases to a position equidistant between them (15).

Mutational studies of *Volvox* by Huskey (17) showed that the individual cellular units must indeed be properly oriented in the colony to enable phototactic behavior. This regular orientation is demonstrably lacking in the *eye* mutants of Huskey (17).

The flagella of an *eye* mutant beat, but as the organisms are unable to move coordinately in any direction, they lose the capacity for phototaxis. It thus appears that the rearrangements of centriole, microtubules, and eyespot are essential and developmentally programmed. Flagella also reorient in *Pleodorina* (12). Assessing the net cost of generating the proper cytoskeleton in an adult *Volvox* awaits an understanding of the underlying molecular events. These events will be clarified by the *Chlamydomonas* genome program, now under way. In any case, the rearrangement of the cytoskeleton is programmed and coordinated with the arrest in cytokinesis. The cost of this set of adaptations is likely to have been significant, and by inference, the advantage of multicellularity must also have been significantly large to offset that cost.

VOLVOX CONCLUSIONS

Despite the costs of the specializations required to make a *Volvox carterii* colony a functional unit, multicellularity arose several times in the genus *Volvox*. Repeat occurrences suggest therefore that selection strongly favors the multicellular state. *Chlamydomonas* must have a cytoskeleton with strict polarity, yet one that permits systematic axoneme rotation. More efficient resource acquisition in eutrophic environments and protection of the offspring and parents from predation could explain most of the major evolutionary trends in *Volvox* and its relatives: increased size, increased cell number, increased matrix volume, and differentiation of germ and soma. The transient abundance of nutrients in quiet ponds each spring followed by late-season scarcity would provide a selective advantage for multicells that could execute phototaxis, photosynthesize efficiently, store precious phosphate against later shortages, and survive an abundance of predators. These advantages combined must have been strong enough to drive the complex cytological and regulatory changes required.

GENERAL CONCLUSIONS

Multicellular cyanobacteria like *Nostoc* enjoy a clear nutritional advantage. The myxobacterial grade of multicellularity has both a feeding advantage and an advantage in dispersion. *Volvox* benefits in feeding by means of its efficient phototaxis, in dispersion, and in protection from predation. Advantages in feeding and in dispersion seem to be common threads in this sample of three organisms.

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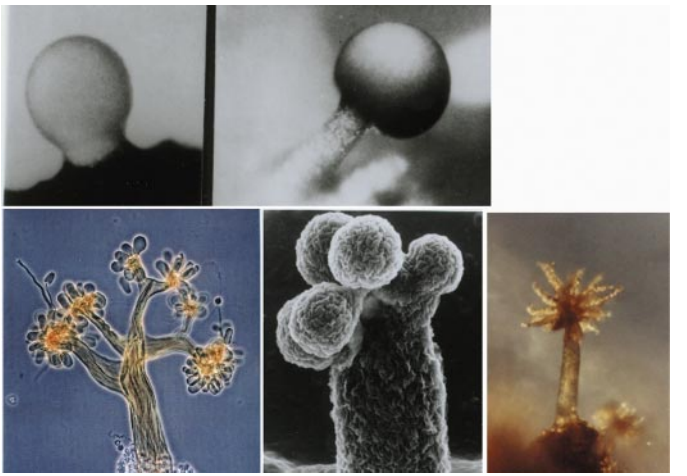

Figure 3 Fruiting bodies (*top row left to right*), *Myxococcus fulvus*, *M.stipitatis*; (*bottom row left to right*), *Chondromyces crocatus*, *Stigmatella aurantiaca*, and *C. apiculatus*. Photographs courtesy Dr. Hans Reichenbach and Dr. Martin Dworkin, University of Minnesota.

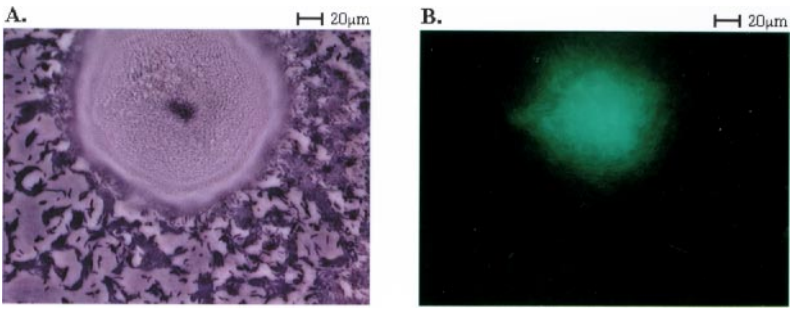

Figure 6 Expression of sporulation genes is localized to the fruiting body. *Panel A*: a single fruiting body photographed from above with visible light. *Panel B*: the same field photographed with ultraviolet light to excite the fluorescence of GFP. Photograph by Bryan Julien (19).

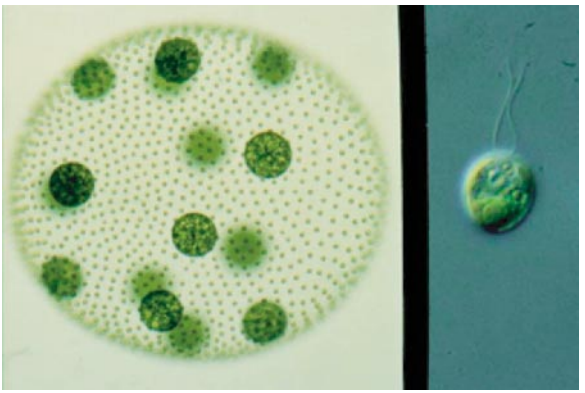

Figure 7 (*Left*) A spheroidal colony of *Volvox carteri*. (*Right*) A single *Chlamydomonas reinhardtii* cell at higher magnification showing its pair of flagella. Photograph courtesy of Dr. David Kirk and Dr. Ursula Goodenough, Washington University, St Louis, MO.