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Cell Differentiation and Colony Alteration of an Edible Terrestrial Cyanobacterium *Nostoc flagelliforme*, in Liquid Suspension Cultures

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ABSTRACT. Morphological characteristics of an edible terrestrial cyanobacterium *Nostoc flagelliforme* in liquid suspension cultures under photoautotrophic conditions are presented. Different cell forms alternated in a regular manner during the experimentation period (30 d). *N. flagelliforme* exhibited a very complex life cycle in terms of colony morphology, including mainly 4 different colony morphological forms, *viz*. hormogonia, filaments, seriate colonies and aseriate colonies. Under laboratory conditions it formed spherical colonies on solid media but not threadlike colonies as it did under natural conditions. The overall life span of the alga was not altered by the existence of different nitrogen sources in the media despite the depression of some cell forms or colony morphologies. Compared with growth on the medium with urea and ammonium as nitrogen sources, the alga on standard medium had a short period of hormogonia and aseriate colony, suggesting that both ammonium and urea could stimulate the formation of hormogonia, at the same time inhibiting the formation of heterocystous cells. The new information on the growth and morphology of *N. flagelliforme* could be potentially used for the scale-up or field cultivation.

Nostoc flagelliforme, formerly *Nostoc commune* var. *flagelliforme*, is an edible terrestrial cyanobacterium of great economic value. Field surveys show that it is distributed on arid or semiarid steppes in many countries including Algeria, China, Czechia, Slovakia, France, Japan, Mexico, Mongolia, Morocco, Russia, Somalia and the USA (Li 1991; Gao 1998). It has been used as a food delicacy for more than two thousand years, and its medicinal value has been recognized since ancient times in China (Gao 1998; Tseng 2001). Its Chinese name, Fat Choi, also includes an auspicious meaning as the characters can be pronounced as a phrase that means "getting rich", which has led to the traditional eating of *N. flagelliforme* as a delicacy on holidays. As a result of the high profits associated with Fat Choi commercial activities, picking and trading of *N. flagelliforme* are now seen as a highly profitable enterprise. However, picking Fat Choi has led to environmental problems such as soil erosion and desertification, resulting in this species becoming endangered to the point of extinction. In 1999, the Ministry of Forestry of China designated *N. flagelliforme* a Category II Protected Plant with the aim of protecting this precious natural resource and the environment. Under the protection regime the picking and trading of wild Fat Choi was strictly prohibited in 2000.

As a supplement to prohibition, however, the Chinese government has encouraged and financially supported many research projects on this alga with a view to enabling artificial cultivation or mass culture of the species. Nevertheless, our knowledge of the biology of *N. flagelliforme* is still fragmentary and we remain far from understanding the biological characteristics of the organism (Gao 1998). Most previous work has focused on the natural mature forms of desiccated colonies, *e.g*., on photosynthetic characterization (Gao and Zou 2001), membrane lipid composition (Wang *et al*. 2000) and polysaccharide composition (Huang *et al*. 1998). However, because of its bacterial and algal nature, liquid suspension culture could be an alternative approach for a high yield production of this species, although basic data on its liquid suspension culture is still lacking.

The objective of the present study was to elucidate the morphological features of *N. flagelliforme* during its developmental span in different liquid suspension cultures under laboratory conditions, including cell differentiation and colony alteration. For the purpose of comparison, observations of growth stages on solid media are also presented and discussed.

MATERIALS AND METHODS

Culture. *Nostoc flagelliforme* strain FACHB-838 was obtained from the *Institute of Hydrobiology, Chinese Academy of Sciences* (Wuhan, China). Axenic culture was maintained in an HBIII culture medium (*Freshwater Algae Culture Collection of the Institute of Hydrobiology* list, *unpublished*; free of nitrogen). Prior to inoculation, the culture was microscopically examined to make sure that hormogonium of *N. flagelliforme* was the dominant form, accounting for more than 95 % of the total cell number. Modified $BG11_0$, nitrogen-free and phosphorus-enriched, were also used.

Inocula (8 %, *V*/*V*) were added to 250-mL Erlenmeyer flasks, each containing 120 mL culture media; all treatments were performed in triplicate. All cultures were incubated axenically in an orbital incubator (2 Hz, 30 °C; *Gallenkamp*, UK) and under continuous illumination provided by an array of cool white fluorescent tubes giving a mean photon flux density of 3 klx at the flask surface. For growth in solid media, *ca*. 0.1-mL inoculum was inoculated onto each agar-solidified BG11 plate.

In order to investigate the effects of different nitrogen sources on the passage through different developmental stages, nitrate in the standard BG11 was replaced by ammonium and urea at the same concentration. The inocula were obtained by repeated subculturing on the media with different nitrogen sources for 3 consecutive batches followed by inoculation into the corresponding culture media (Attridge and Rowell 1997).

During a 30-d experiment, morphological characteristics of the cultures were examined using a *Leica* DM RXA versatile microscope equipped with a Wild MPS48 camera system.

RESULTS

Cell differentiation. Fig. 1 shows five identifiable cell forms, *viz*. vegetative cell (Fig. 1D, VC), heterocyst (Fig. 1B, C, HC), proheterocyst (Fig. 1C, E, PHC), akinetes (Fig. 1B, AK) and germling (Fig. 1F, GE). All five forms alternated in a regular manner during liquid suspension culture. Vegetative cells usually dominated at the onset of culture (Fig. 1D), the increase in the biomass being mainly due to their vigorous division. In standard BG11 medium, the vegetative cells began to differentiate into proheterocysts or heterocysts. Some intercalary heterocysts occurred at different interheterocyst spacing in N-free media ($BG11_0$ and HBIII). Cell differentiation into akinetes commenced, indicating that cells grew in suboptimal conditions, such as the depletion of nutrients and light attenuation due to the increased viscosity of the culture. The akinetes were either randomly distributed in the absence of heterocysts or, in the presence of heterocysts, they were located close to their neighboring terminal or to intercalary heterocysts. Degenerate heterocysts also appeared as empty cells, which eventually disintegrated or became detached from the filaments, resulting in filament fragmentation. Individual akinetes were released by the senescent colonies to await optimal conditions for germination. Under optimal conditions, akinetes germinated to several hormogonia *via* the formation of germlings (Fig. 1F).

Colony morphology on agar plates. *N. flagelliforme* can easily colonize and develop a wide range of macroscopic colonies that have different colors, shapes, sizes and textures on agar plates (Fig. 2). The size of the spherical colonies (or pearls) ranged from 0.5 to 5 mm, with a majority of 1–2 mm. Along with the progression of growth on the plates, there was a color change from light green (Fig. 2A, B) to dark green or dark (Fig. 2C, D). Microscopic observation of these ball-like colonies revealed that early colonies were completely packed with long filaments, each with more than 20 vegetative cells and 2–5 heterocysts, while the mature colonies (Fig. 2D) consisted of relatively short filaments and amorphous substances that were assumed to be exopolysaccharides (Potts 2000). Due to physical constraints, further colonial development ceased.

Colony morphology in liquid suspension culture. A distinct life cycle can be observed in the forms of the various colony appearances on the culture media. At the onset of incubation, vegetative cells tended to prevail (Fig. 3A). These formed hormogonia of different lengths, with 7–15 cells within each hormogonium. After a short period of growth (1–2 d), two changes took place, *viz*. the formation of proheterocysts and heterocysts, and the rapid transverse and longitudinal division or budding (Fig. 3B, C). At the same time, the colony turned deep green in color due to the concomitant growth of the vegetative cells during this stage, which corresponded to the peak in the growth curves (*data not shown*). Consequently, cell division mainly occurred in the longitudinal plane, leading to the gradual disappearance of the filamentous character. In addition, cells increased in size and tended to be granulated. The next stage was marked by the formation of spiral aggregates (Fig. 3D). The whole colony appeared to be contorted and separated into several compartments, each containing 5–10 cells. Moreover, the colonies at this stage were characterized by the presence of external polysaccharide investment in the form of capsules or gelatinous sheaths or envelopes. As a result,

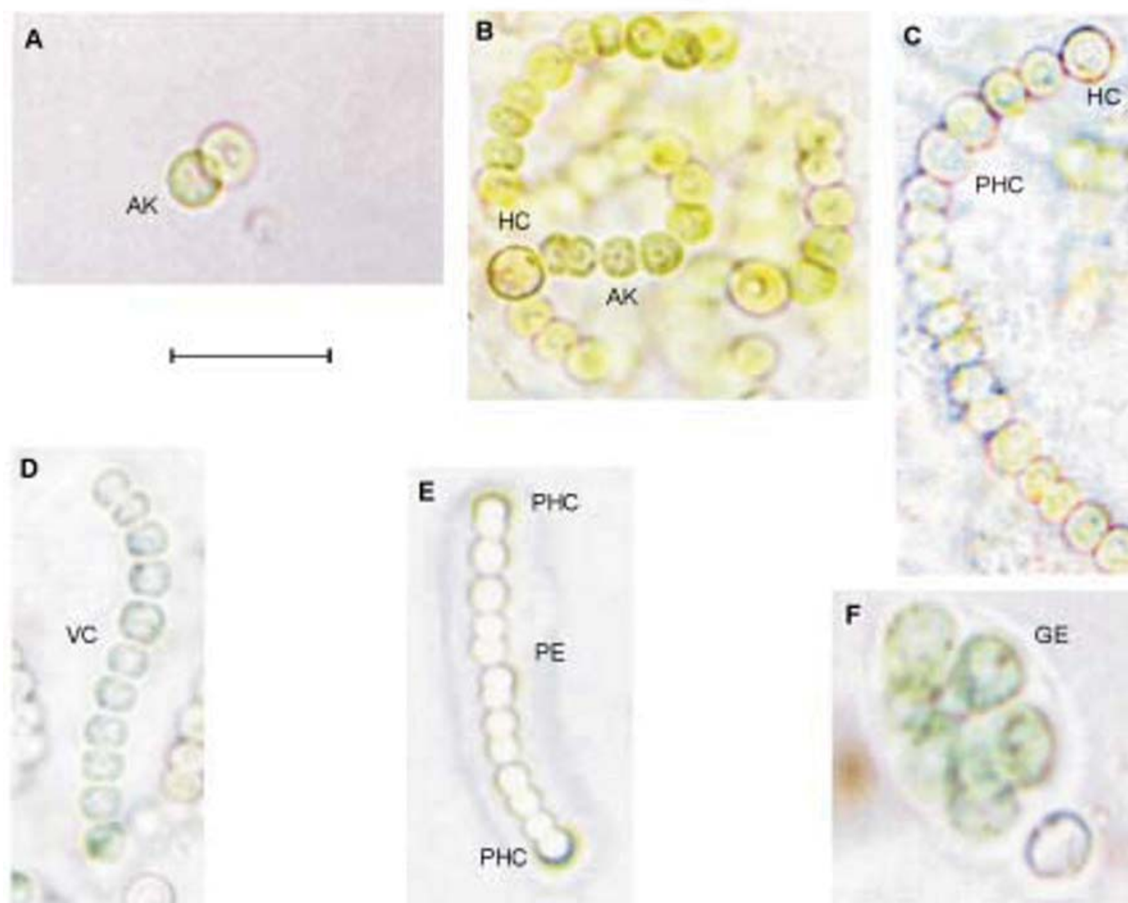

Fig. 1. Microphotographs of *N. flagelliforme* cells showing cell differentiation. **A**: Germinating akinetes (AK); **B**: co-existence of akinetes and heterocyst (HC); **C**: a terminal heterocyst and an intercalary proheterocyst (PHC); **D**: a string of vegetative cells (VC); **E**: hormogonium with two terminal proheterocysts encapsulated in a gelatinous pellicle (PE); **F**: further germinating akinetes with many germlings (GE); *bar* = 20 µm.

akinetes occurred in large quantities with a few heterocysts appearing along the outer edge. Finally, the colonies were fragmented into many small colonies in aseriate forms (round, ellipsoid, *etc*.) followed by the detachment of akinetes (Fig. 3E). As a result of the germination of the akinetes, a few motile hormogonia were found (Fig. 3E, F). The whole culture became yellowish because of the breakdown of the larger colonies and the subsequent release of some hydrophilic photosynthetic accessory pigments (*not shown here*). This stage was therefore characterized by the co-existence of the germinating akinetes, short and motile hormogonia, and capsulated larger colonies (Fig. 3H). An overall picture of the life span of *N. flagelliforme* in liquid culture is presented in Fig. 4: Four different developmental stages in the life span can be identified, *viz*. hormogonia, filament, seriate colony, and aseriate colony.

Effect of different nitrogen sources on developmental stages was also shown (Fig. 5). Although the 4 developmental stages might overlap, or occurred simultaneously in *N. flagelliforme*, these growth stages could still be distinguished from each other. Compared with medium BG11 with urea and ammonium as nitrogen sources, this species in standard BG11 and BG110 had a relatively short period of hormogonia and a long duration in which it appeared a as seriate colony, suggesting that both ammonium and urea might stimulate the formation of hormogonia, while at the same time inhibiting the formation of heterocystous cells. It is worth noting that there seemed to be no difference between standard BG11 and $BG11_0$ in terms of the duration of each developmental phase, which indicated that the effect of nitrate was neutral.

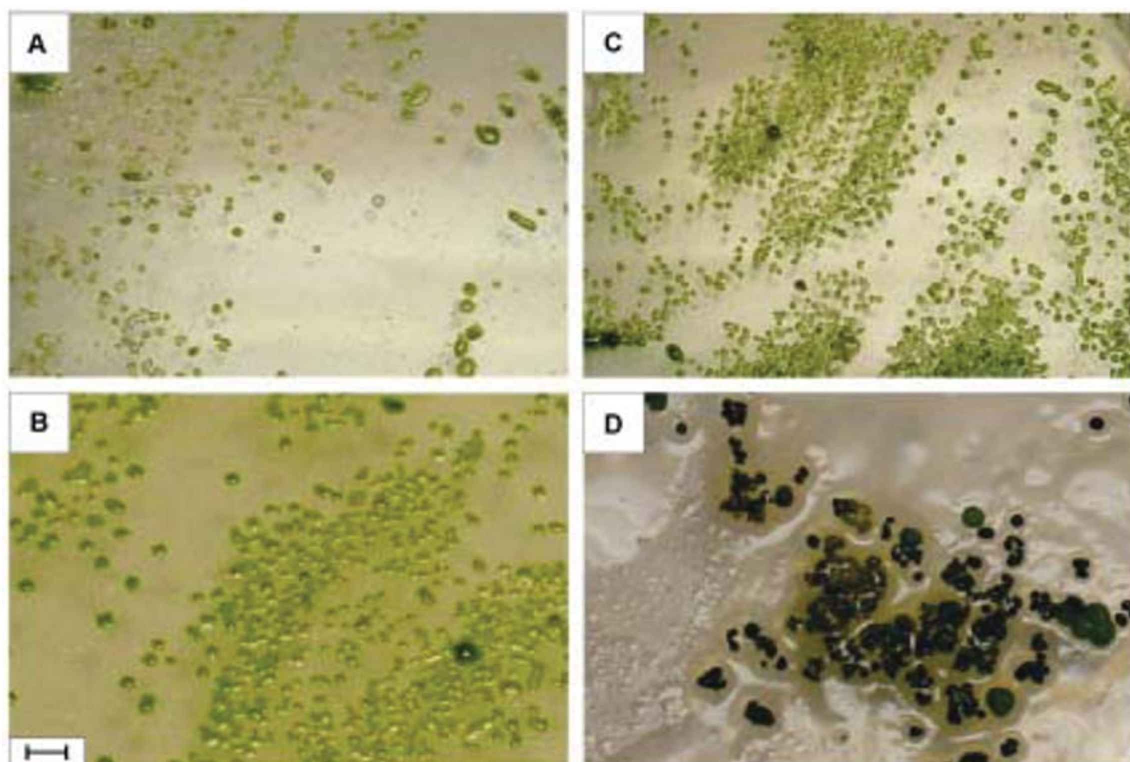

Fig. 2. Microphotographs of *N. flagelliforme* colonies on agar solidified BG11 plates; **A**–**D** arranged in chronological order; *bar* = 10 mm.

DISCUSSION

Cell differentiation, especially heterocyst differentiation, which is regarded as a response to changes in physical, chemical or biological conditions, is very common in cyanobacteria under both natural and laboratory conditions (Agrawal and Singh 2000, 2002; Agrawal and Misra 2002; Agrawal and Pal 2003). Among many factors which influence this process, nitrogen and phosphorus are critical. The mechanism by which these factors cause the vegetative cells to become highly specialized remains unclear. Our results were consistent with previous findings that proheterocyst or heterocyst formation was depressed by the nitrogen source in the form of nitrate, and that the nitrogen-fixing condition (diazotrophic condition) favored the formation of heterocysts (Adams and Duggan 1999). However, ammonium and urea appeared to retard further development along a normal life cycle (cf. Fig. 5).

We showed that the life cycle of *N. flagelliforme* is quite similar to those of *N. commune* strains UTEX 584 and DRH1 (Potts 1994, 2000), which undergo a 4-stage process, in which the forms of hormogonia, filaments, spiral aggregates (seriate colonies) and aseriate colonies (or pearl colonies) predominate. The difference lies in the position of heterocystous cells and perhaps also in the number of heterocysts. At the stage of hormogonia and filaments, there appear to be more heterocystous cells in *N. commune* than in *N. flagelliforme* and the formation of intercalary heterocysts is very vigorous in *N. commune* while in *N. flagelliforme* the intercalary heterocysts are relatively rare. Another point is that interheterocyst spacing is much lower in *N. commune* than in *N. flagelliforme*. As pointed out by Adams and Duggan (1999), the necessary interchange of nutrients between heterocysts and vegetative cells dictates that the heterocysts develop in positions that ensure the most efficient production and distribution of fixed nitrogen to the vegetative cells. The relationship between the number of intercalary heterocysts and the interheterocystous spacing suggests that there is a difference at the molecular level in the control of heterocyst development and spacing, as the same key genes and proteins are involved in heterocystous organization (Adams 2000).

Colonial development on plates failed to progress into cyanobacterial mats or darkened crusts like those that we had observed in nature. Several factors may be responsible for this phenomenon. As observed by Scherer and Zhong (1991), the characteristic strands of this species covered exposed rocks or limestone regions in semi-arid areas, such as the Gobi Desert in Inner Mongolia (China), suggesting that this organism showed a preference for alkaline environments in terms of pH requirement and also that high calcium con-

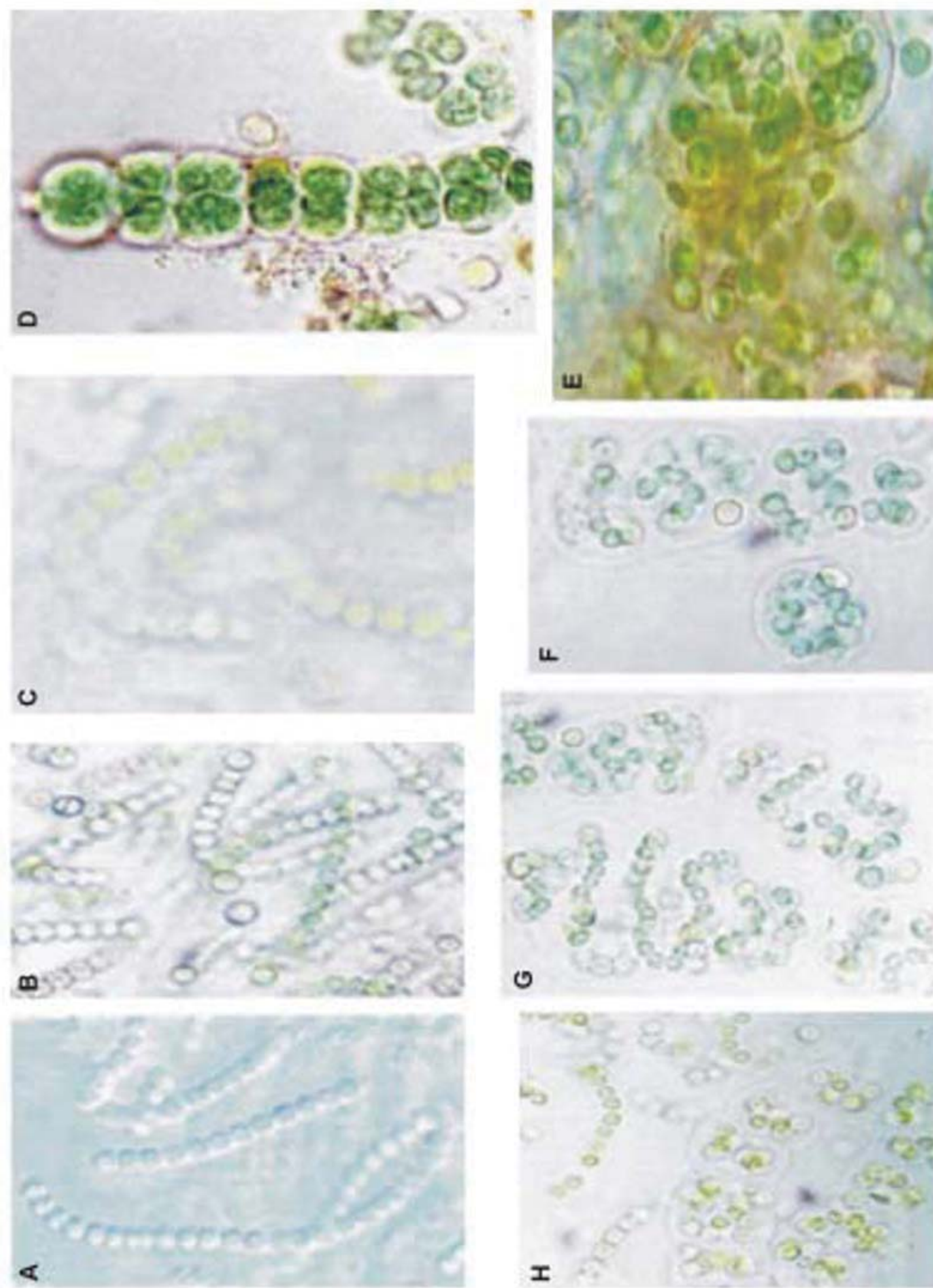

Fig. 3. Microphotographs of *N. flagelliforme* colonies, demonstrating the proceeding of some representative developmental stages. **A**: young hormogonia with rare heterocysts ; **B**: filaments with some heterocysts; **C**: vigorously growing filaments; **D**: seriate colonies that seem to be contorted, cells beginning to divide in both the transverse and longitudinal plane; **E**: colonies breaking down into small compartments; **F**: small aseriate (ball-like) colonies with akinetes and heterocysts, encapsulated in a gelatinous sheath; **G**: further development of the ball-like colonies, more heterocysts and akinetes appearing; **H**: akinetes germinating into short, motile trichomes, leading to the co-existence of hormogonia, seriate and aseriate colonies; magnification in A–E – ×40.

centrations favored this species during its growth. Alternating conditions of precipitation and dryness could be another factor that made the crust visually conspicuous. However, the growth conditions leading to the formation of crust were not provided in this paper (Scherer and Zhong 1991). Colonies on both agar plates and in nature could be pigmented, with coloration ranging from dark green to black or yellow green to reddish brown. This was probably related to the secretion of specific substances such as scytonemin (Potts 2000), but the precise mechanism remains unknown.

According to Anand and Revathy (1993), it should be possible for the specific factor responsible for akinete germination to be unequivocally determined in an experiment under different environmental conditions. Our experiments, however, indicated that the triggers for akinete differentiation could be light limitation and nutrient depletion, in particular phosphorus starvation. This finding was consistent with observations in *Anabaena cylindrica* (Nichols and Adams 1982; Herman 1987, 1988) and was further supported by our observation that, in the presence of excess phosphorus in the medium (modified $BG11_0$), akinetes continued to

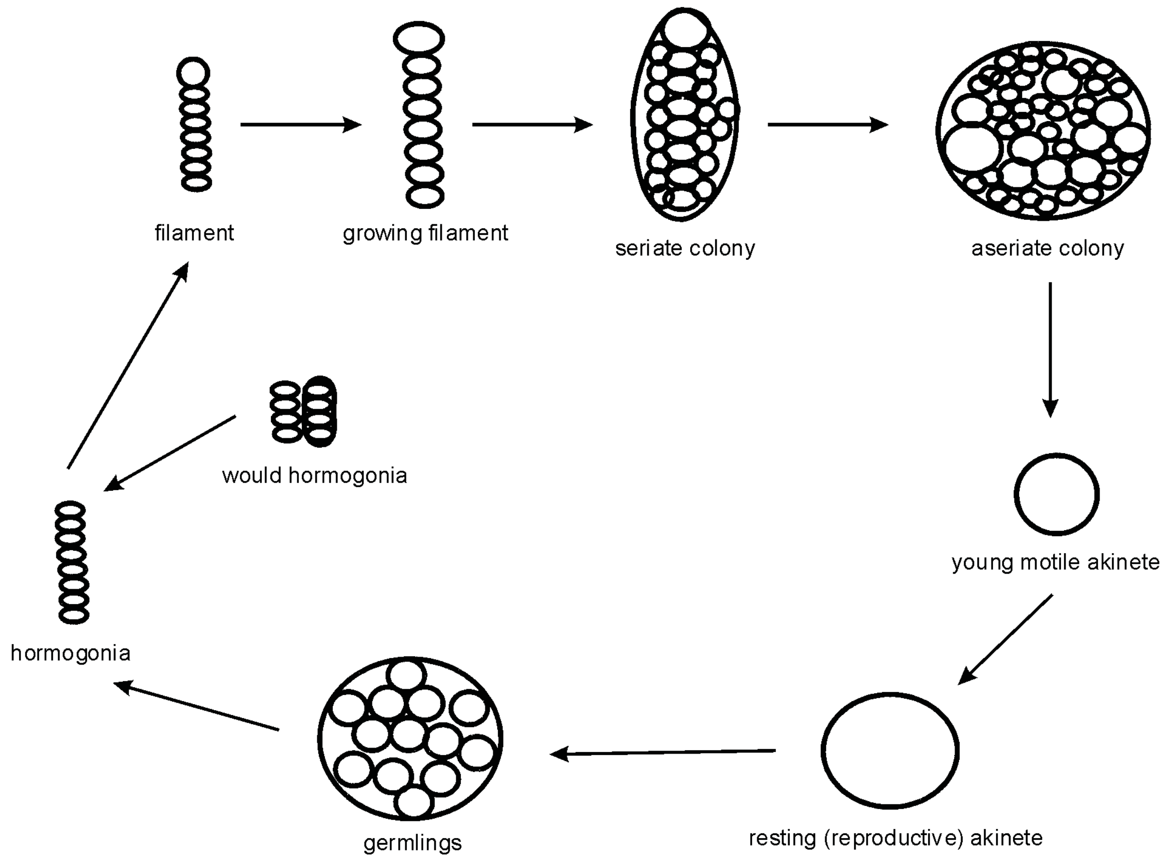

Fig. 4. Scheme of the complex life span of *N. flagelliforme*, indicating that its development can be divided into 4 phases: hormogonia, filament, seriate colony and aseriate colony; the large cells are designated as heterocystous or proheterocystous cells, the small cells as vegetative cells, and the medium-sized cells as akinetes.

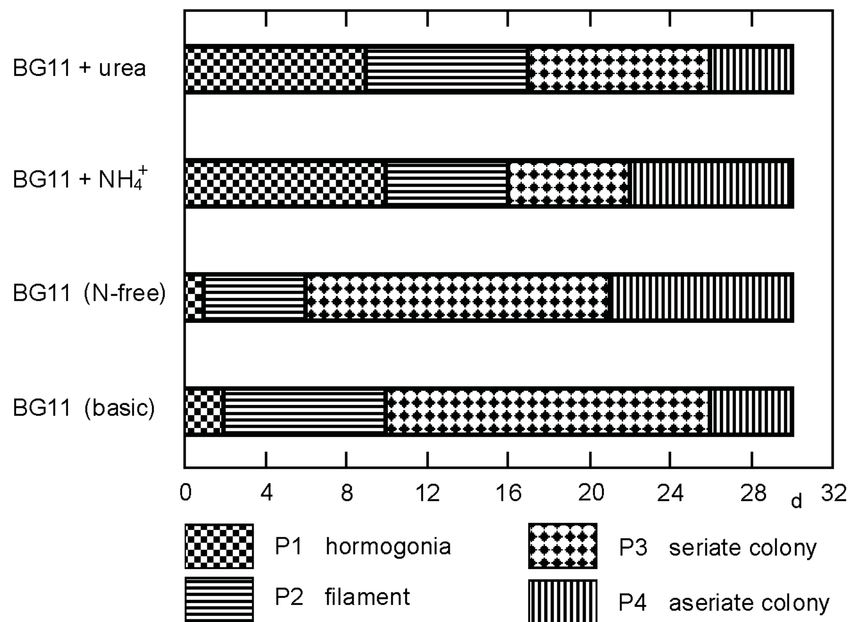

Fig. 5. Progression of cell and colony morphology of *N. flagelliforme* in modified BG11 media – without nitrogen, with nitrate (basic medium), with ammonium or urea (instead of nitrate) as nitrogen source; the entire life cycle consists of 4 developmental phases, *viz.* hormogonia (P1), filament (P2), seriate colony (P3) and aseriate colony (P4).

differentiate even when light was limited as a result of cell mutual shading at high cell concentration. Phosphorus, and perhaps a wide variety of other nutrients as well, could be implicated as a trigger in akinete development for this alga. For some species of *Anabaena*, however, temperature or a critical C : N ratio appeared to be more important factors (Li *et al.* 1997; Sarma and Khattar 1993).

Another similar study (De Philippis *et al.* 2000*a*) offered a general scheme of the developmental cycle of 40 exopolysaccharide-producing *Nostoc* strains with special emphasis on trichome (reproductive filament) morphology. A discrepancy between that study and the present one was that our results showed that in the stages of hormogonia and filament, equivalent to the unpellicled shape stage described by De Philippis *et al.* (2000*a*), the trichomes were seldom surrounded by exocellular mucilaginous investments. We also showed that in the seriate and aseriate stages, the exopolysaccharide-surrounded colonies prevailed, suggesting that vigorous secretion of exopolysaccharide by *N. flagelliforme* occurred in the later developmental stages.

Recently, the majority of *Nostoc* species have come to be regarded as promising sources for the production of polysaccharides both in dried colonies and liquid suspension cultures (Huang *et al.* 1998; De Philippis and Vincenzini 1998; De Philippis *et al.* 2000*a*,*b*). As a member of the genus *Nostoc*, *N. flagelliforme* is also characterized by the presence of external polysaccharidic investments that are often released into the surroundings as water-soluble polysaccharides. Unfortunately, there have been no systematic studies of the relationship between the production of exopolysaccharides and cell differentiation in the *Nostoc* species. To our knowledge, cell differentiation of *N. flagelliforme* appeared to be repressed by an appreciable excretion of expolysaccharides and the subsequent self-inhibitory effects. In this study, the hormogonia or wound hormogonia were often prevalent in the colony morphology and cells could not proceed to become specialized while the viscosity of the aqueous solution was increasing in some culture (*unpublished data*).

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