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EXPERIMENTAL ARTICLES

Baeocytes in the Cyanobacterium *Pleurocapsa* sp.: Characterization of the Differentiated Cells Produced by Multiple Fission

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Abstract—Electron microscopy of cyanobacterium *Pleurocapsa* sp. CALU 1126 revealed that multiple fission proceeds by successive binary fissions. The cultivation conditions were determined when the number of baeocytes (products of multiple fission) was comparable with that of macrocytes (products of binary fission), and cell sorting was achieved for the first time. Juvenile baeocytes were shown to differ from macrocytes in: (1) the absence of sheath; (2) the linear-peripheral configuration of their lamellar system; (3) lower content of phycobiliproteins and higher content of carotenoids; (4) the set of PSII polypeptides. Baeocytes can therefore be considered differentiated cells characterized by the uncoupling between energy and biosynthetic metabolism.

Key words: cyanobacteria, multiple fission, baeocyte.

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Bacterial cells usually undergo binary fission. *Citromicrobium bathyomarinum* [1] and *Pelodictyon* spp. [2], capable of ternary fission, and *Deinococcus* spp., which are characterized by unique tetrary fission [3], are exceptional.

In the hyphae of mycelial or sporogenous actinobacteria [4], a number of septa (transverse and sometimes also longitudinal) can be formed simultaneously. In this case, replication processes and cytokinesis precede growth; this type of division is termed multiple fission.

Apart from higher actinobacteria, cyanobacteria of the order *Pleurocapsales* (Subsection II) are capable of multiple fission [5]. Binary fission coupled to cell growth results in the formation of macrocytes (big sheathed mother cells). Multiple fission not coupled to growth results in the formation of a sporangium, i.e., a rudimentary sheath filled with baeocytes (small daughter cells with or without sheaths). After liberation from the sporangium, the baeocytes develop to macrocytes.

The cytological mechanism of multiple fission in cyanobacteria is not clear. A series of sequential binary fissions of a macrocyte [5] and multiple fission are the theoretical possibilities. Multiple fission in *Dermocarpa* sp. was reported in 1984 [6]; these findings have not been either confirmed or refuted. The baeocytes have not been characterized, apart from their (in)capacity for gliding movement, depending on the absence or presence of the sheath [5].

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The present work deals with the mechanism of multiple fission in cyanobacteria exemplified by *Pleurocapsa* sp. CALU 1126. Moreover, the results of the newly developed method of cell sorting suggest that baeocytes can be considered differentiated cells.

MATERIALS AND METHODS

Enrichment culture. Strain *Pleurocapsa* sp. CALU 1126 was isolated in 1999 from the water of the Lake Ladoga euphotic zone [7]. The culture was maintained in flasks or test tubes with liquid or solid (0.8% of Difco Bacto Agar) $BG11^M$ mineral medium [8] at ambient temperature and continuous illumination with white fluorescent lamps (0.5×10^3 lx).

Pleurocapsa sp. CALU 1126 grows more slowly than the heterotrophic contaminants which penetrate into the sheathed aggregates of macrocytes and sporangia. Our attempts to obtain an axenic culture by means of serial dilutions or exhausting streak inoculation in the presence of cycloheximide (100 μg ml^{-1}, Serva) and chloramphenicol (20 μg ml^{-1}, Serva) were not successful. Similar attempts to obtain axenic cultures from the sorted baeocytes were thawed by their poor survival on solid $BG11^M$ medium. In the case of cultivation in the vessels described below, the level of contamination revealed by direct microscopic counts was ≤5%.

Tests for baeocyte motility. The standard procedure [9] was used to determine the baeocyte (in)capacity for gliding motility. The stationary-phase cells from

liquid culture were placed on an agarized plate. Motile baeocytes exhibit positive phototaxis under such conditions. The locations of free baeocytes were determined under a dissection microscope.

Cell sorting. To obtain individual populations of macrocytes and baeocytes, the cells were cultivated in 500-ml cylindrical airlift vessels with 300 ml of BG11^M medium, at 23°C and continuous illumination with white fluorescent lamps (1.5×10^3 lx). Sterile air was supplied at the rate of 100 ml min^{-1}. The peak of baeocyte formation ~3.5×10^6 cells ml^{-1} was detected after 14–16 days. Macrocytes, sporangia, and free baeocytes were present in the culture simultaneously.

The mixture of macrocytes and sporangia was precipitated at 100 *g* for 1 min; the baeocytes remaining in the supernatant were precipitated at 1000 *g* for 10 min. Sporangia were disintegrated by resuspending the macrocytes/sporangia pellet in 5 mM phosphate buffer (pH 8.0) and sonication for 15 s at 5°C Techpan UD-20 (Poland). Intact macrocytes and empty sporangia sheaths were precipitated at 1000 *g* for 1 min, and the baeocytes in the supernatant at 1000 *g* for 10 min. The efficiency of cell sorting was verified microscopically; the baeocyte fraction contained trace amounts of macrocytes and empty sporangia sheaths, while the fraction of macrocytes and sporangia sheaths contained trace amounts of baeocytes.

The biochemical analysis was carried out immediately or after storing the material at –70°C.

Microscopy. Light microscopy was carried out using a Leica DRMXA phase contrast microscope (×1500). The microphotographs were obtained with differential interference contrast (Nomarsky) optics and a Leika digital camera using the Leika IM50 software package.

For electron microscopy, the material was prefixed for 1 h with 0.25% OsO_4 at 4°C, washed with 5 mM phosphate buffer (pH 8.0), and fixed for 2 h at 4°C with 2.5% glutaraldehyde (Sigma) in 5 mM cacodylate buffer (pH 8.0). The sample was washed twice with 5 mM phosphate buffer (pH 8.0), postfixed for 12 h with 1% OsO_4 in the phosphate buffer, washed with phosphate buffer, dehydrated in increasing concentrations of ethanol and in acetone, and embedded in Spurr (70°C, 15 h). Ultrathin sections (~300 Å) were prepared on a Reichert ultratome. The sections were contrasted with uranyl acetate/lead citrate and studied under a JEOL JEM-100 microscope at 9 kV.

Pigment analysis. In order to determine the specific content of chlorophyll *a* and total carotenoids, the baeocyte cells were extracted with methanol (pH 8.0) at ambient temperature and centrifuged at 1000 *g* for 5 min. Extinction of the supernatant was determined on a Beckman DW spectrophotometer at 666 and 476 nm ($E_{666} = 0.072$ for chlorophyll *a* and $E_{476} = 0.220$ for total carotenoids [10]).

In order to obtain the light fraction of the cell-free preparation, enriched with thylakoid membranes baeocyte cells were resuspended in 0.5×10^3 mM Na phosphate buffer (pH 8) and disintegrated by 60-s sonication. After centrifugation for 5 min at 5×10^3 *g*, the supernatant contained the light fraction. Pigment extinction in this fraction was determined on a Beckman DW spectrophotometer in the wavelength range of 350–700 nm. In order to identify the pigments, the characteristics of their absorption spectra were compared to those in the literature [8].

Electrophoresis of membrane polypeptides. The protein sample for electrophoretic separation was obtained by precipitation from the light fraction of the cell-free homogenate at 2×10^4 *g* for 15 min. The membrane phospholipids and pigments were extracted from the sediment with ethanol–ether (3 : 1), chloroform–methanol (2 : 1), and ether. The polypeptides were then solubilized for 3 min at 100°C in 5×10^{-2} M Tris–HCl buffer (pH 6.8) (Serva), supplemented with SDS (Serva), 10%; 2-mercaptoethanol (Serva), 5%; and glycerol (Serva), 20%. Electrophoresis was carried out according to Laemmly in 15% polyacrylamide gel (Serva) at 4×10^{-2} A; the 20–66 kDa set of molecular mass (Serva) was used.

RESULTS AND DISCUSSION

The pleurocapsalean cyanobacteria (Subsection II, phylum BX *Cyanobacteria*) reproduce (exclusively or at a certain stage of their life cycle) by means of baeocytes, unique reproductive cells [5]. Due to the difficulties of maintaining the cultures of these slowly growing bacteria with asynchronous acts of binary and multiple fission, these organisms are scarcely represented in collections and poorly studied.

Macrocyte morphology. In the case of *Pleurocapsa* sp. CALU 1126, we did not detect the pseudofilamentous structures characteristic of the pleurocapsalean cyanobacteria with more complex morphology [5]. Fission of macrocytes along several planes results in formation of irregular aggregates, stabilized by sequentially growing sheaths (Fig. 1). Apart from a gram-negative cell wall, the macrocytes are always surrounded with a cover layer, which structurally corresponds to the fibrillar sheath of a presumably polysaccharide nature found in other cyanobacteria (Fig. 2a). Unlike the cell wall, it does not participate in binary fission. The sheath formation is induced along the whole surface of both daughter cells. As the individual fibrillar layers thicken, the daughter cells become separated, and the mother cell sheath stretches.

Conditions of baeocyte formation. The physiological or ecological factors directly inducing multiple fission are not known. In laboratory conditions asynchronous multiple fission was shown to commence 12–24 h after the transfer of a stationary-phase culture into a

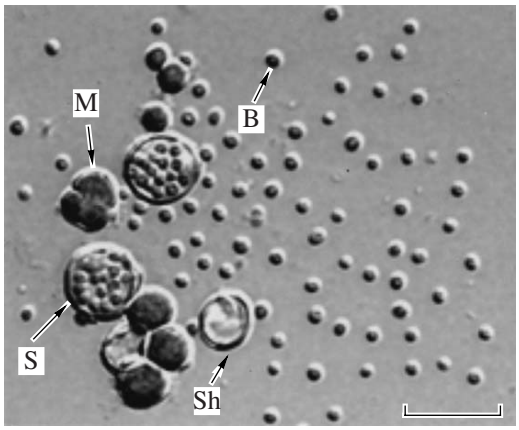

Fig. 1. Light microphotograph of *Pleurocapsa* sp. CALU 1126. Scale bar, 10 μm. M, macrocyte; S, sporangium; B, baeocyte; Sh, sporangium sheath; T, thylakoid; V, blebbing vesicles.

fresh medium [11]; the ratio of the acts of binary reproduction and multiple fission is known to depend on the mode of cultivation [5].

Comparative study of the cellular organization of baeocytes and macrocytes required the cultivation mode which enabled their simultaneous accumulation. We have previously determined [7] that the relative frequency of acts of multiple fission decreases with increasing temperature within the range of 20 to 30°C; a temperature of around 23°C was the optimum for baeocyte formation. Since *Pleurocapsa* sp. CALU 1126 grows slowly on solid media, forming cell aggregates closely attached to the gel surface, we used submerged cultivation in vessels of significant volume with continuous stirring; these conditions stimulated more rapid cyanobacterial development and prevented cell adhesion to the cultivator wall. The peak of baeocyte formation under these conditions was observed on the 14th–16th day of cultivation. Multiple fission of a single macrocyte resulted in the formation of 2^5–2^6 baeocytes (Fig. 1).

Mechanism of multiple fission and baeocyte ontogenesis. Multiple replication of the macrocyte chromosome occurs prior to multiple fission [11]. Dependence possibly exists between the number of sister chromosome copies and the number of septae. The character of prebaeocyte division (binary or multiple fission) is, however, still a debatable question [5, 6]. The exact direction of sister chromosome movement is in turn possibly determined by attachment of the replication origins to fixed loci of the cytoplasmic membrane.

In *Pleurocapsa* sp. CALU 1126, we observed no evidence of multiple fission by the formation of a series of radial or tangential septae [6]. Binary fission, however, was observed (Fig. 2b, c); thus, multiple fission occurs very rapidly and via an alternative pathway.

The applicability of transmission electron microscopy to detailed analysis of the cytokinesis program is limited. Intravital light microscopy with the hybrid fluorescent reporter FtsZ-GFP is the most adequate approach to the study of sister nucleoid compartmentalization [12].

Binary fissions are partially synchronized during the early stage of fission; the few large prebaeocytes form tetrads (Fig. 2c). At a later stage, sporangia contain numerous small baeocytes (Fig. 2d).

The baeocytes are surrounded by an amorphous, electron-dense material of unknown composition (Fig. 2c, d). In accordance with earlier observations [13], the baeocytes are liberated from the sheath due to its mechanical rupture (Fig. 1), rather than enzymatic lysis. When released from sporangia spontaneously or by ultrasound treatment, the baeocytes initially have no sheath (Fig. 2e). In the course of maturation, baeocytes are covered with a sheath (Fig. 2f) and transformed to macrocytes.

Thus, multiple fission in *Pleurocapsa* sp. CALU 1126 is based on rapid sequential fissions of a macrocyte; the mother cells (prebaeocytes) do not grow between the fissions. Binary fission, unlike multiple fission, is always preceded by growth of a mother cell. The growth program is evidently blocked at the genetic level; the search for the corresponding master regulator is an independent problem.

Characteristics of differentiated cells observed in baeocytes. In *Pleurocapsa* sp. CALU 1126, the diameter of juvenile baeocytes (i.e. those in sporangia or immediately after liberation) is several times smaller than that of macrocytes (Fig. 1; Fig. 2d, e); their volume is two orders of magnitude smaller. These significant differences in size are accompanied by differences in their ultrastructure and biochemical characteristics.

The absence of a sheath is the first indication of baeocyte differentiation (Fig. 2b–e). Sheath biosynthesis is suppressed in the course of multiple fission; for a short time (6–24 h), free baeocytes may exhibit gliding motility [13], while macrocytes are always nonmotile. Baeocytes exhibit positive phototaxis: in a gradient of light, they move to the region with optimal illumination [9]. Loss of motility occurs simultaneously with initiation of the biosynthesis of the fibrillar sheath; the cells then start to grow and transform to macrocytes. In our case, baeocytes initially had no sheath (Fig. 2e), although their motility has not been detected. Experimental conditions possibly did not favor phototactic behavior; more probably, however, juvenile baeocytes lose motility rapidly, since the sheath formation commences immediately after their liberation from the sporangium (Fig. 2f). This agrees with the appearance of the blebbing vesicles (Fig. 2e), which are believed to be associated with secretion [14]. Since baeocyte motility is among the characteristics included in the taxonomic

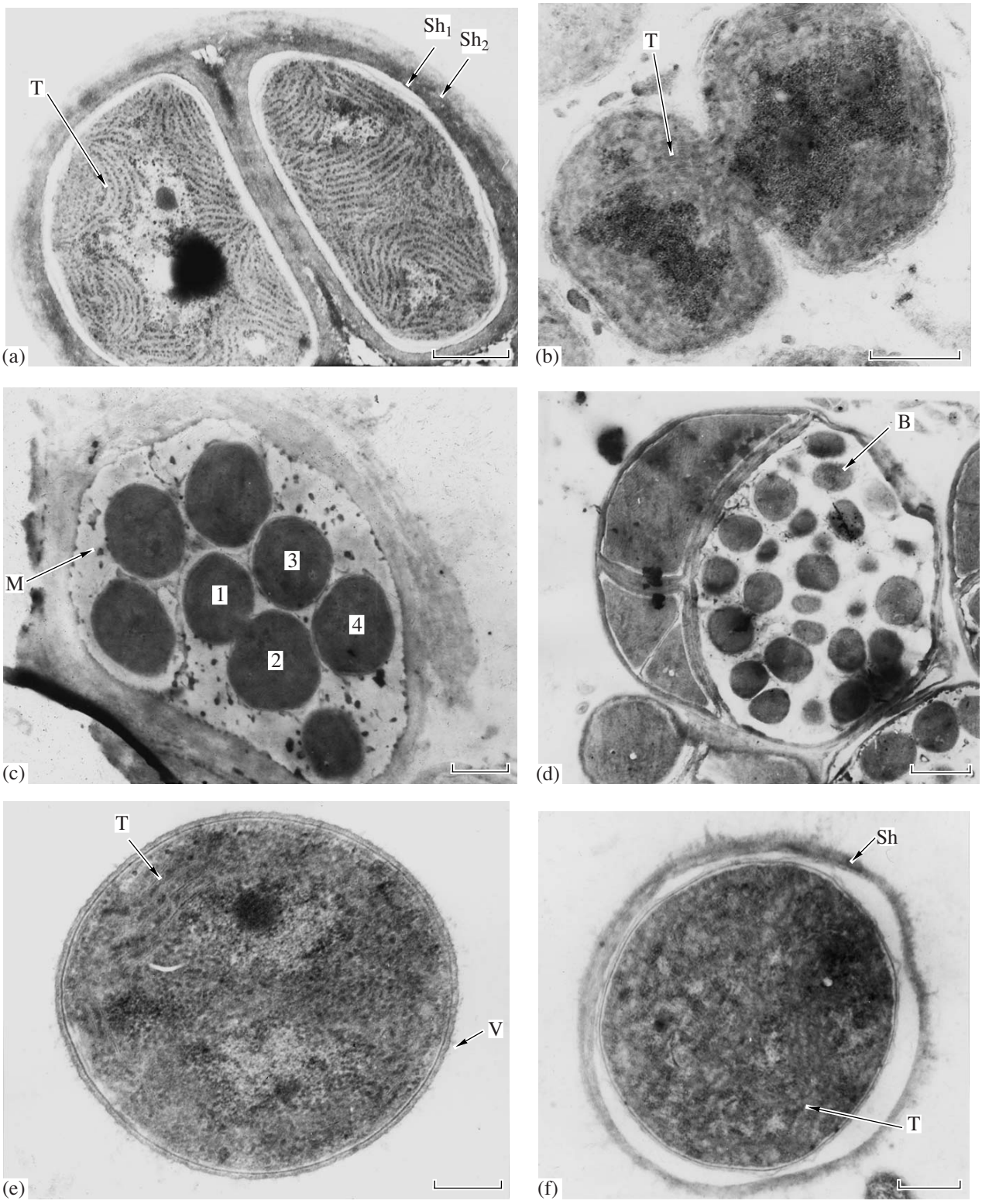

Fig. 2. Electron micrographs of *Pleurocapsa* sp. CALU 112 ultrathin sections: (a), macrocytes; (b), binary division of a probaeocyte; (c), early stage of multiple fission; (d), late stage of multiple fission; (e), free baeocyte without a sheath; (f), free sheathed baeocyte. Scale bars: 2 μm (d); 1 μm (a, c); 0.5 μm (b); 0.2 μm (e, f). Designations as on Fig. 1.

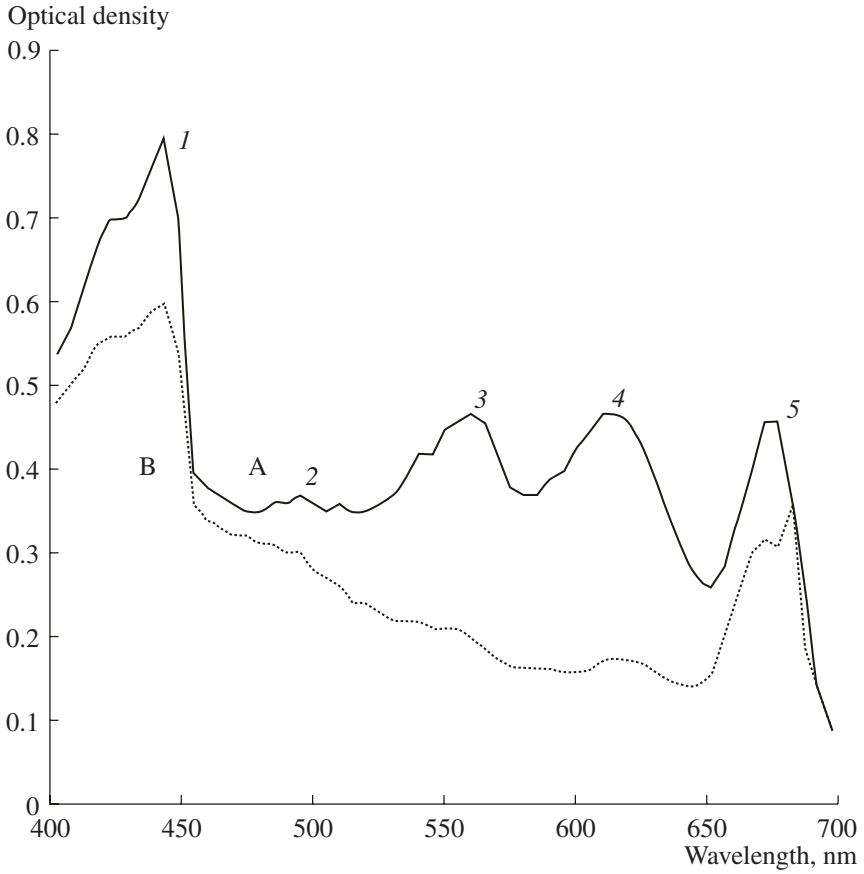

Fig. 3. Absorption spectrum of *Pleurocapsa* sp. CALU 1126 cell-free preparations: macrocyte preparation A and baeocyte preparation B. Maxima: *1* (440 nm) and *5* (675 nm), chlorophyll *a*; *2* (500 nm), carotenoids; *3* (570 nm) phycoerythrin; *4* (630 nm), phycocyanin.

description of the order *Pleurocapsales* [5], our results may indicate a necessity of its revision.

Content of hydrophobic pigments (μg mg^{-1} of dry biomass) in *Pleurocapsa* sp. CALU 1126 (average values; $n = 3$)

Pigment(s)	In macrocytes	In baeocytes
Chlorophyll *a*	4.71*	9.26
Carotenoids	0.89*	3.63
Carotenoids: chlorophyll *a*	0.19	0.39

Note: Content of chlorophyll *a* and carotenoids in macrocytes is an underestimate, since the sheaths contribute to the biomass increase.

Baeocytes also differ from macrocytes in the size, topology, and morphology of their lamellar system. The macrocytes are uniformly filled with concentric layers of curved thylakoids (Fig. 2a). The lamellar system of the forming baeocytes consists of peripheral thylakoids oriented in parallel to the cell envelope (Fig. 2b). This configuration of the lamellar system is retained in juvenile baeocytes (Fig. 2e).

Moreover, baeocytes contain less phycobiliproteins and more carotenoids than macrocytes (Figs. 3 and 4 and table). This finding indicates the changed qualitative ratio between integral antennae and the main light-harvesting complex (phycobilisome).

Finally, baeocytes and macrocytes differ in their set of membrane polypeptides (Fig. 5). Apoproteins of PSI (66, 17–3 kDa), PSII (47, 43, 34, 32, 26, 20, 17–4 kDa), and of the cytochrome *bf* complex (32, 23, 20, and 17 kDa), as well as ATP synthase subunits (54, 35, 30, 17–6 kDa) are known to dominate among the latter [15]. Within the range of polypeptides with apparent molecular mass of ~70 kDa (skeleton apoproteins of the PSI reaction center), no differences were revealed between baeocyte and macrocyte preparations. The dif-

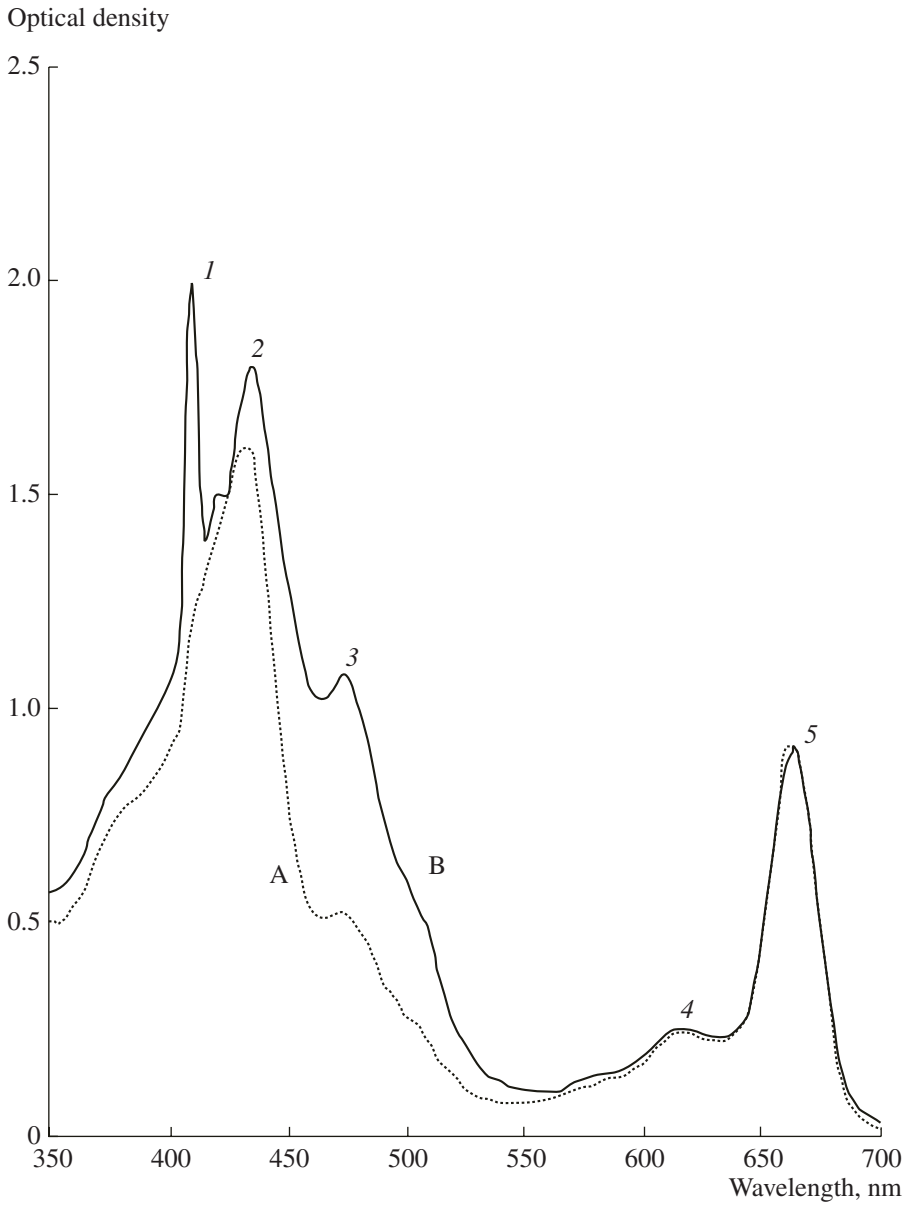

Fig. 4. Absorption spectra of *Pleurocapsa* sp. CALU 1126 methanol extracts: macrocyte preparation A and baeocyte preparation B. Maxima: *1* (410 nm) and *3* (470 nm), carotenoids; *2* (430 nm), *4* (620 nm), and *5* (665 nm), chlorophyll *a*.

ferences between electrophoretic spectra were most pronounced for the polypeptides with apparent molecular mass of 30–33 and 35–45 kDA (skeleton apoproteins of the PSII reaction center and core antenna); their content was higher in baeocyte preparations.

Thus, baeocytes exhibit characteristics of differentiation, in particular those related to the surface structures and the photosynthetic apparatus.

Similar changes occur in the course of akinete and heterocyst differentiation [16]. Although adaptive strategies of these three types of differentiated cell forms are different, all of them share uncoupling of the energetic and constructive metabolism.

The biological meaning of multiple fission is evident: it provides for intensified reproduction and extensified spreading [17, 18].

The biological sense of the peculiarities of the photosynthetic apparatus in the baeocytes is also evident. Multiple fission is related to division; it, therefore, occurs at a higher rate than the biosynthetic reactions performed by PSII and its antenna (phycobilisome) [15]. Juvenile baeocytes require energy (supplied mostly by PSI), rather than biosyntheses. Although

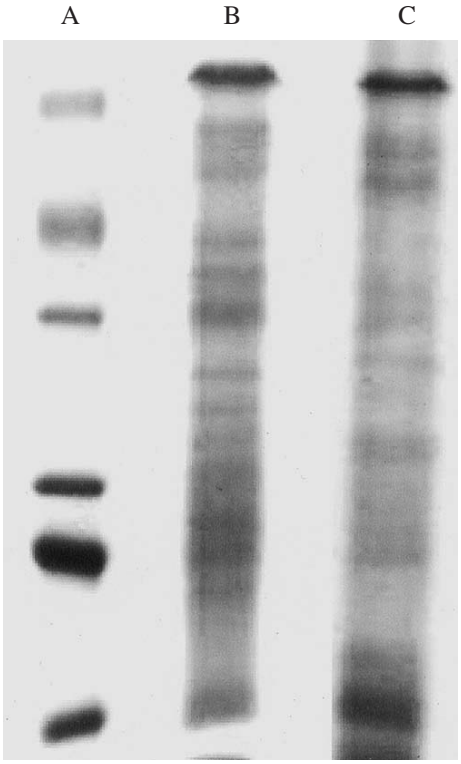

Fig. 5. Electrophoregram of *Pleurocapsa* sp. CALU1126 membrane polypeptides (after staining with Coumassie-R250): A, molecular mass standards (20–66 kDa); B, baeocyte preparation; C, macrocyte preparation.

decreased phycobiliprotein content should affect the PSII activity, accumulation of its apoproteins may be explained by a preparatory state, which ensures their transition to active growth.

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