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Epifluorescence microscopy of *Anabaena* sp.: nucleoid configurations and evidence for inclusion-associated DNA

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Abstract. Vital staining of the nucleoid in *Anabaena* sp. PCC7118 was performed using the double stranded DNA-specific fluorochrome DAPI. In the unicellular mutant, the central epifluorescent zone had a dense skein configuration, while in the filamentous parent strain protrusions, lobes, and distinct isolated elements of the nucleoid were visible. Both variants contrasted with the mainly peripheral, partitioned structure typical of plastids and prochlorophytes. Blue-white emittance from the DNA-DAPI complex was maximum in dividing cells, suggesting that DNA configuration is linked to the cell cycle events. In stationary cultures, epifluorescent cell inclusions were conspicuous: based on this observation, we argue that DNA is associated with carboxysomes in situ.
Key words. Cyanobacterial nucleoid epifluorescence; inclusion-associated DNA.

The genetic apparatus of prokaryotes (bacteria and archaea) is far less extensively studied than its counterpart in the eukaryotic cell. Cyanobacteria are among the largest and most differentiated bacteria, but their genophore arrangement is still difficult to define. These phototrophs are especially rich in internal membranes and may prove to be an exception to the chromosome-cell envelope attachment dogma[1]. In cyanobacteria, the chromosome seems to be associated with thylakoids[2], and the resemblance to plastids[3] is of considerable evolutionary interest. The in situ observation of DNA-containing regions contributes to our understanding of the cyanobacterial chromosome dynamic structure and function. Electron microscopy of thin sections requires careful interpretation and is especially prone to artifacts, so staining with DNA-specific dyes may prove a more reliable indicator of structure. Unfortunately, this has previously been performed on fixed and extracted cells[3–6].

In contrast, we report here on vital epifluorescence microscopy of *Anabaena* sp. PCC7118. Our prime interest was the nucleoid configuration: the evidence for inclusion-associated DNA was obtained in the course of the study.

Materials and methods

Axenic cultures of *Anabaena* sp. PCC7118 were used throughout this study. A spontaneous trichome non-forming mutant (Ucl, from 'unicellular'), similar to the parent strain in pigmentation and growth rate[7], was initially used for reasons of homogeneity and focussing convenience. Both strains were grown autotrophically in thermostated airlift vessels in Allen's medium, supplemented with 0.1% sodium thiosulfate to prevent possible harmful effects of oxygen radicals, at 25 °C with constant white-light irradiation of 25 μmol photon $m^{-2} \cdot s^{-1}$. Cultures were grown to an approximate cell density of $2 \cdot 10^7$ ml^{-1}, corresponding to the exponential growth phase; stationary cultures with an approximate cell density of 10^8 ml^{-1} were also used. When necessary, exponential cultures were synchronized by a 24 h dark-incubation, followed by constant illumination, as above. Synchronization was monitored by direct cell counting in a Petroff-Hauser chamber. Stationary, asynchronous exponential, dark-pretreated, and synchronized cultures were centrifuged at $3 \cdot 10^3$ rpm and gently resuspended in neutral 50 mM sodium phosphate buffer. Aliquots were incubated under the growth conditions for 1 h with 5 μg · ml^{-1} 4′, 6-diamidino-2-phenyl indole (DAPI) and 1 mg · ml^{-1} dimethyl sulfoxide (both Serva), collected by centrifugation and mounted in 5 mg · ml^{-1} agarose (Sigma A grade) directly on specimen glasses. Stained samples were observed with a Carl Zeiss Standard Photomicroscope equipped with an epifluorescence attachment and a × 100 phase contrast objective, using phthalic acid dibutyl ester (Serva) as the non-fluorescent immersion liquid. The preparations were excited with a 200 W mercury lamp through a UV-filter (350 nm). Epifluorescence micrographs were taken with a high sensitivity X-ray negative film; phase micrographs were taken of the same field.

Results and discussion

Visualization of cyanobacterial nucleoids with DAPI has conventionally been based on epifluorescence microscopy of fixed and ethanol/acetic acid-bleached

cells[3,8]. In contrast, our staining procedure was performed without any fixative or extractant; the presence of the mildly permeabilizing agent dimethyl sulfoxide is known not to interfere with the physiological states of bacteria[9] or their internal structure. Special attention was given to the reproducibility of emittance intensity that proved to be highly dependent on cultural conditions (see below). The use of unfixed, virtually intact cells meant excluding DNAse treatment, a customary control in epifluorescence studies[5]. Our indirect control criteria to insure the artifactual nature of our observations were: 1) the use of a generally accepted DNA-specific fluorochrome in relatively low concentrations, 2) the bright blue-white colour of the emittance typical of DNA-DAPI complex, and 3) the spatial correspondence of the emitting zone to nucleoplasm.

Application of very low concentrations of DAPI ($0.1-0.5\ \mu g \cdot ml^{-1}$) resulted in a weak, rapidly fading epifluorescence. With the higher concentration recommended[3] ($5\ \mu g \cdot ml^{-1}$), a strong stable fluorescence was observed. In our case, DAPI-treated cells were essentially devoid of the chromatoplasm background epifluorescence which is due to the dye binding non-specifically to thylakoids. Pigment autofluorescence, interfering with or completely masking DAPI-staining, was also absent. Correspondingly, unlike previous reports on the subject, we could abandon glutaraldehyde fixation and acidic ethanol bleaching, conventionally used in experiments with DAPI-staining[4].

Typically, after prolonged (15 min or longer) exposures to the exciting beam, the chromatoplasm produced red emittance indicative of UV-induced damage of the photosynthetic apparatus. However, exposures of 1.25 min (which was optimum for taking epifluorescence micrographs) caused no decline in viability, as demonstrated by the colony-forming units counting test performed on 2% Difco Bacto-Agar plates with Allen's medium and 0.1% sodium thiosulfate (data not shown).

Nucleoid configurations were primarily studied in exponential cultures. In dividing cells of the unicellular mutant (fig. 1a and 1c), centrally-located, discrete epifluorescent zones could be seen; they were completely (fig. 1b) or partially (fig. 1d) distributed between the daughter cells. Here the nucleoid occupied the bulk of the cell interior, had a dense skein appearance, and was not partitioned into separate fluorescent masses. It should be noted that the epifluorescence images possibly represented the pattern of intact nucleoid, since the superimposition of phase and epifluorescence micrographs demonstrated spatial correspondence between the transparent (fig. 1a) and the brightly emitting (fig. 1b) zones. In fast-growing filaments of the parent strain, various stages of nucleoid partitioning were visible (fig. 1e). Here, in contrast to the mutant, discrete epifluorescent zones (fig. 1f) or a continuous zone with thin protrusions and massive lobes (fig. 1g) could be seen. This discrepancy in nucleoid configurations might be associated with the thylakoid distribution: a chaotic one in the mutant vs an organized one in the parent strain[7]. Paired phase/epifluorescence micrographs in figure 1 and figure 3 also show that, although the presence of undetectable thin protrusions of the nucleoid could not be ruled out[2], the epifluorescent zone had no visible contacts with the cell margins.

Despite profound differences in staining procedure, our pictures were very similar to those obtained with fixed preparations of cyanobacteria[3,10]. With the single exception of epizoic *Synechocystis* sp. (which was shown to have an atypical, disperse nucleoid[8]), cyanobacteria have a central, compact nucleoid arrangement which differs from that in plastids and prochlorophytes[5,6,11] (whose nucleoids were an irregular epifluorescent mass distributed among thylakoids in the organelle/cell periphery).

A variable and still rather weak epifluorescence was observed in non-dividing cells of exponential (fig. 3f and 3h) and of stationary (fig. 4b, 4c, and 4d) cultures. As represented by figure 4b, the epifluorescence was dispersed throughout the cytoplasm; the isolated zone of the nucleoid was absent. The association of maximum nucleoid epifluorescence with the D period of the cell cycle was not evident until relatively homogeneous populations of dividing cells were studied. To verify whether maximum epifluorescence was consistently associated with division, we compared pre-synchronized and synchronized cultures of the parent strain.

Among cyanobacteria only unicellular strains, *Synechococcus* sp. and *Synechocystis* sp., have been reported to be synchronized[12,13]. The cell cycle of *Synechococcus* sp. PCC6301 ('*Anacystis nidulans*') was studied in detail. Exponential cultures placed for 12–24 h in the non-permissive dark conditions were shown to arrest with incomplete cell cycles. Restoring the illumination initiated a novel cycle resulting in synchronous division in 60–95% of cells[12]. In our case, the light/dark/light shift was first applied to the filamentous cyanobacterium. The partially synchronous growth pattern was demonstrated by the limited fraction of dividing cells (approx. 75%), as well as by the long time (approx. 4 h) necessary to achieve the peak in cell number (fig. 2). Here the synchronization was followed for only one division period and defined by nearly the same parameters[12,13] as those in *Synechococcus* sp. PCC6301 and *Synechocystis* sp. CALU752, suggesting a similar control of the replication systems. Typically, dark-pretreated, non-dividing cells gave a faintly visible epifluorescence (fig. 3b). In contrast, synchronously dividing cells had brightly emitting nucleoids (fig. 3d). In trichomes of the asynchronous exponential culture (fig. 3e and 3g), the completion of cell divisions (shown by constrictions, septi, and full partitioning) correlated with the decline of nucleoid emittance (fig. 3f and 3h).

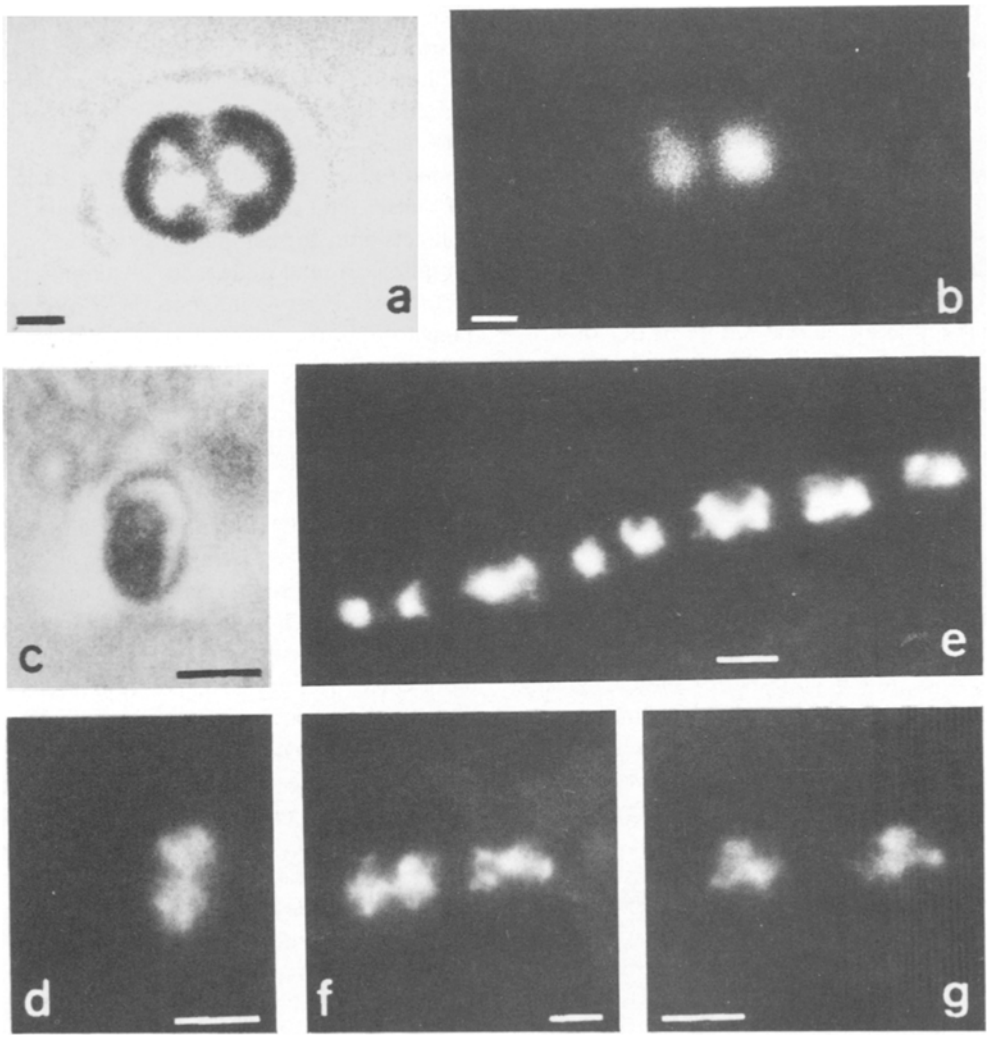

Figure 1. Phase and epifluorescence micrographs of DAPI-stained *Anabaena* sp. PCC7118, unicellular mutant (a–d) and filamentous parent strain (e–g). Asynchronous cultures. Scale bar, 1 μm (a, b) and 5 μm (c–g).

Presumably, different intensities of the nucleoid epifluorescence reflected different states of chromosomes in dividing and in non-dividing cells. Compacting bacterial DNA or supercoiling domains are supposed to show dynamic transients in vivo[14]. This idea was corroborated by the characteristic condensed appearance of the nucleoplasm in electron micrographs of dividing *Aphanothece* sp.[15], and was indirectly supported by the reduced epifluorescence of intermitotic nuclei in DAPI- or Hoechst 33258-treated *Chlamydomonas reinhardtii*, *Pandorina morum* and *Volvox carteri*[4]. Although our data are necessarily descriptive and allow several interpretations, they indicate that nucleoid organization may be associated with specific cell cycle events. There is no doubt that fluorochrome staining performed at different points in the cell cycle is a promising, albeit not very sensitive, indicator of nucleoid dynamic structure.

Besides the above data on nucleoid configurations, DAPI-staining gave evidence of an association between DNA and cell inclusions: unicellular mutant in stationary cultures contained up to three brilliant blue-white bodies (fig. 4), clearly distinct from the nucleoid. Results with the parent strain were identical and are not shown. According to their 300–500 nm-size, hexagonal (?) shape (fig. 4d, bottom) centroplasmic location and occurrence in cells of the stationary culture, these inclusions probably corresponded to carboxysomes[16,17]. As demonstrated by figure 4a and 4b, the epifluorescent inclusion has an opaque equivalent in the phase micrograph. In our case, artifactual staining was unlikely due to the specific blue-white colour of the epifluorescence, unlike, for instance, the orange-yellow colour of staining due to polyphosphates[5]. In general, the above data may be interpreted as indicative of an in situ association between DNA and carboxysomes. Earlier observations with cyanobacteria or cyanellae[3,18] gave no evidence of DAPI binding to the 'polyhedral bodies' or any other cell inclusions.

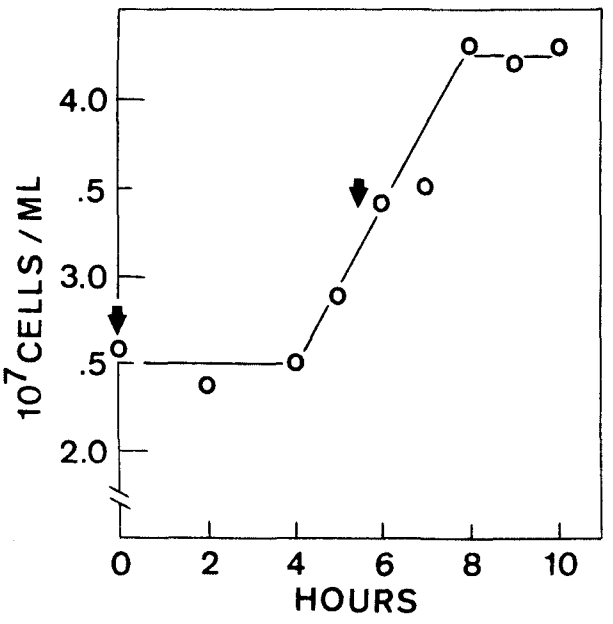

Figure 2. Growth curve of synchronized *Anabaena* sp. PCC7118, filamentous parent strain. Light re-exposure after 24 h dark-pretreatment. Arrows indicate the removal of samples for microscopy.

Currently, the in situ association of DNA with prokaryotic cell inclusions is thought to be restricted to carboxysomes. This is supported by: 1) the ubiquitous localization of 'polyhedral bodies' to the (peri)nucleoid zone[17], 2) the phosphorus-positive signal of cyanobacterial carboxysomes as determined by the electron spectroscopic imaging (ESI) method[19], 3) the presence of DNA in purified carboxysome preparations of some nitrifiers and thiobacilli[20,21], and 4) the complex role ascribed to carboxysomes in inorganic carbon (C_I) concentration/fixation/reduction that makes them functionally analogous to the genophore-bearing organelles (hence, calvinosomes)[22]. The most convincing evidence, 3), was originally considered to be an indication of the genetic autonomy of carboxysomes[21]. However, restriction endonuclease cleavage showed this not to be the case: electrophoretic mobility patterns coincided for carboxysome-bound and for chromosomal DNAs[20]. The chromosome-carboxysome complex, if present in situ, may have a specific, yet-to-be-defined function. In particular, DNA proximity or binding to the carboxysome may be due to the short lifetime(s) of mRNA(s) coding for certain functional component(s) of the C_I-utilizing system. For instance, it was recently suggested that carbonic anhydrase might exist in a carboxysome-immobilized form[16]. Complex morphological relationships[15] between DNA fibrils, ribosomes and 'polyhedral bodies' makes this speculation more plausible.

Finally, our data provide the first evidence of nucleoid configurations in vitally stained rather than fixed and depigmented cyanobacteria, and of a correlation between nucleoid maximum epifluorescence with cell division. Our observations on the in situ association between DNA and cell inclusions may be considered with earlier data[20,21] on DNA present in isolated carboxysomes. Future application of an immunocytochemical method to colocalize nucleoid DNA and specific

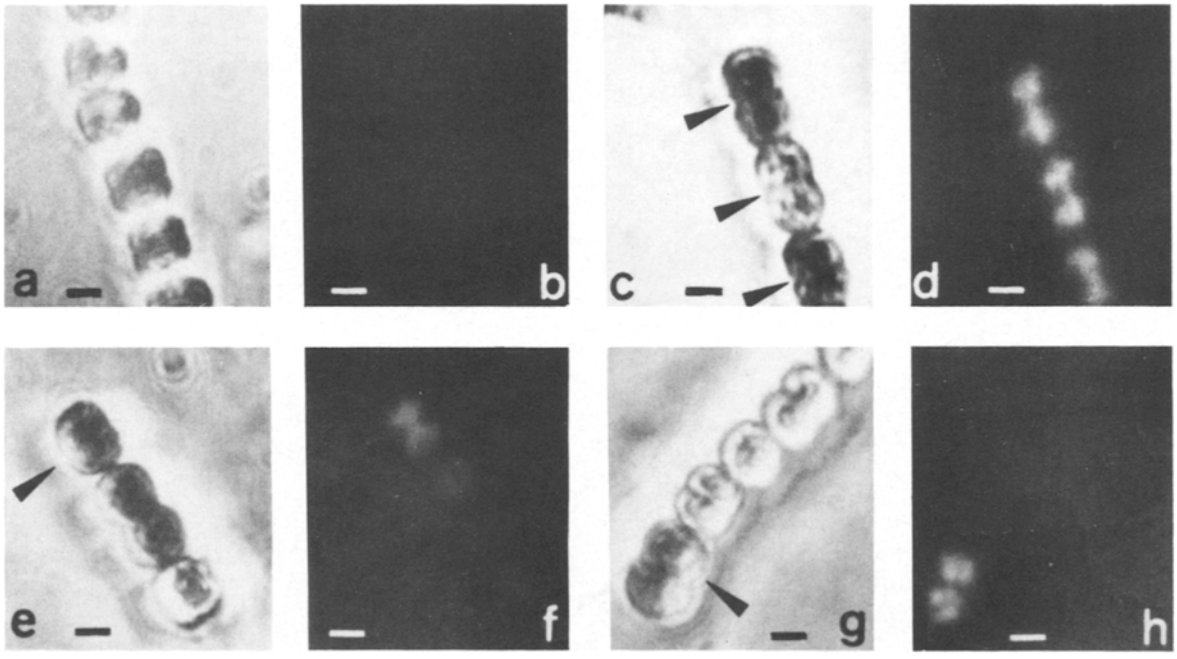

Figure 3. Phase and epifluorescence micrographs of DAPI-stained *Anabaena* sp. PCC7118, filamentous parent strain. Dark-pretreated (a, b), synchronized (c, d), and asynchronous exponential (e–h) cultures. Arrows indicate dividing cells. Scale bar, 5 µm.

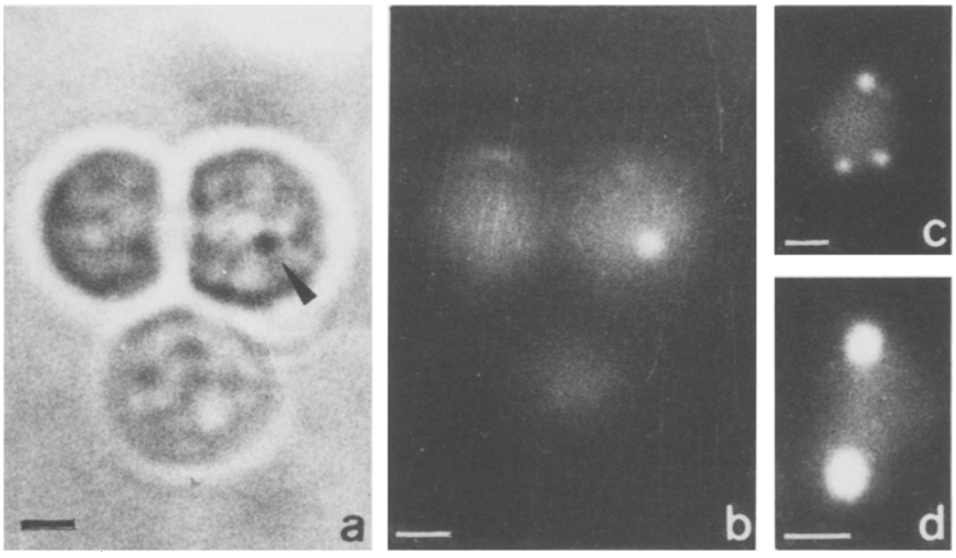

Figure 4. Phase and epifluorescence micrographs of *Anabaena* sp. PCC7118, unicellular mutant. Stationary culture. Arrow indicates the presumed carboxysome. Scale bar, 1 μm.

carboxysome components (especially α and β subunits of RuBisCo) should prove of interest in this respect.

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