Arch Microbiol (1995) 163:447–453

ORIGINAL PAPER

Xavier Ariño · Jose-Julio Ortega-Calvo
Mariona Hernandez-Marine · Cesareo Saiz-Jimenez

Effect of sulfur starvation on the morphology and ultrastructure of the cyanobacterium *Gloeothece* sp. PCC 6909

Received: 26 October 1994 / Accepted: 1 March 1995

Abstract *Gloeothece* sp. PCC 6909 is a unicellular, nitrogen-fixing cyanobacterium that accumulates sulfate in its sheath. An ultrastructural study of sulfate-deficient and normal *Gloeothece* sp. PCC 6909 cells was carried out. The physiological alterations, caused by sulfur starvation, were related to important morphological alterations in the cell: a structureless sheath, accumulation of cyanophycin, polyhydroxybutyrate and glycogen granules, and disintegration of thylakoidal membranes. Most of these changes were reversed by the addition of sulfate to the culture medium. The important role of sulfate in the sheath structure was demonstrated.

Key words Sulfur starvation · Cyanobacterium · Sulfate · Sheath · *Gloeothece*

Introduction

Gloeothece sp. PCC 6909, a unicellular cyanobacterium capable of fixing nitrogen aerobically, is surrounded by a mucopolysaccharide sheath with several concentric layers. It has been speculated that the function of this sheath in cyanobacteria is to provide a controlled microenvironment and to concentrate essential nutrients that are present at submarginal levels (Lange 1976).

Chemical analysis of the *Gloeothece* sheath reveals neutral or acidic polysaccharides and usually a considerable amount of protein (Weckesser et al. 1987; Tease and Walker 1987). Also, sulfate residues are present in the sheath of *Gloeothece* sp. ATCC 27152 (Tease and Walker 1987). A considerable accumulation of sulfur in the concentric sheath layers has been reported in *Gloeothece* sp. PCC 6909, and a possible role of sulfur in sheath formation has been suggested by Tease et al. (1991). However, the sulfate present in the sheath is not available for growth. Recently, Ortega-Calvo and Stal (1994) have shown that the sulfate depletion in batch cultures of *Gloeothece* sp. PCC 6909 due to its incorporation in the polysaccharide sheath causes bleaching, a slow growth rate, and low nitrogenase activity. In continuous cultures, cells do not bleach because of the constant input of sulfate present in the culture medium. The temporal segregation between nitrogen fixation and oxygenic photosynthesis during light/dark cycles occurs in sulfur-starved cells, while in sulfate-sufficient batch cultures and in sulfate-limited continuous cultures, nitrogenase activity is confined mainly to the light period (Ortega-Calvo and Stal 1994).

Considering the importance of sulfur nutrition for a basic knowledge of the physiology of this cyanobacterium, the aim of the present study was to characterize the changes in morphology and ultrastructure of *Gloeothece* sp. PCC 6909 cells during sulfur starvation, which to our knowledge has not been reported previously.

Materials and methods

Organism and growth conditions

Gloeothece sp. strain PCC 6909 was obtained from the Pasteur Culture Collection (Paris, France). The organism was grown in 300 ml of BG 11° medium, which was devoid of any source of combined nitrogen (Rippka et al. 1979), either without sulfate or with 1 g $CaSO_4$. The dissolution of $CaSO_4$ constitutes a continuous input of sulfate to the medium and provides enough sulfate for balanced growth and sheath sulfatation in this organism. The culture was aerated with prefiltered compressed air and maintained at room temperature (22 ± 2° C) for 9 days, after which a sample was withdrawn and centrifuged for transmission electron microscopy (TEM). The culture was illuminated by white cool circular fluorescent tubes (Philips TLD 18W/54), giving an incident irradiance of 35 μE m^{-2} s^{-2}.

X. Ariño · J.-J. Ortega-Calvo · C. Saiz-Jimenez (✉)
Instituto de Recursos Naturales y Agrobiologia,
C.S.I.C., Apartado 1052, E-41080 Sevilla, Spain
Fax +34-5-4624002
e-mail saiz@cica.es

M. Hernandez-Marine
Laboratori de Botanica, Facultat de Farmacia,
Universitat de Barcelona, Avinguda Joan XXIII s/n,
E-08028 Barcelona, Spain

Electron microscopy

Specimens fixed with a mixture of glutaraldehyde (2.5%) and paraformaldehyde were postfixed with 1% osmium tetroxide in the same buffer. They were dehydrated by a graded acetone series, embedded in Spurr's resin (Sigma, Alcobendas, Spain), sectioned at 70 nm with an Ultracut E (Reichert, Nussloch, Germany), and the sections were stained with 2% uranyl acetate and lead citrate. A more detailed description of the procedure for manipulation of cells is given by Hernandez-Marine (1992). Sections were examined in a Philips 200 electron microscope. The procedure used for ruthenium-red-staining has been described by Vaara (1982).

Unstained thick sections (ca. 0.50 μm) of Spurr-embedded samples were placed on 200 mesh formvar copper grids, which do not interfere with sulfur detection by energy-dispersive analysis. Sections were stabilized with a thin carbon film and examined using a Hitachi-800 MT electron microscope operating at 100 kV in scanning-transmission mode (STEM). For the detection and localization of sulfur accumulations within the cells, areas were analyzed by energy-dispersive microanalytical techniques, using a Kevex quantum analytical spectrometer interfaced with the microscope. The generated X-ray maps of sulfur were collected with a Si(Li) detector over a 26-min counting period.

Analytical pyrolysis

Pyrolysis was performed in a Fisons gas chromatograph/mass spectrometer model GC 8000/MD 800 coupled to a Fischer 0316 Curie-point pyrolyser, using a 30 m × 0.25 mm SPB-5 column. The GC oven was held at 25° C with a cryogenic unit and programed to 280° C, at a rate of 5° C/min. The final temperature was held for 20 min. The Curie-point temperature was 500° C.

Results

General morphology

Gloeothece sp. PCC 6909 cultures containing sulfate rapidly increased in cell density, becoming blue-green. Cultures without sulfate began to bleach after day 4 and remained in a stationary phase, with no apparent growth. Observations using a light microscope revealed considerable morphological differences (Fig. 1). Cells grown in

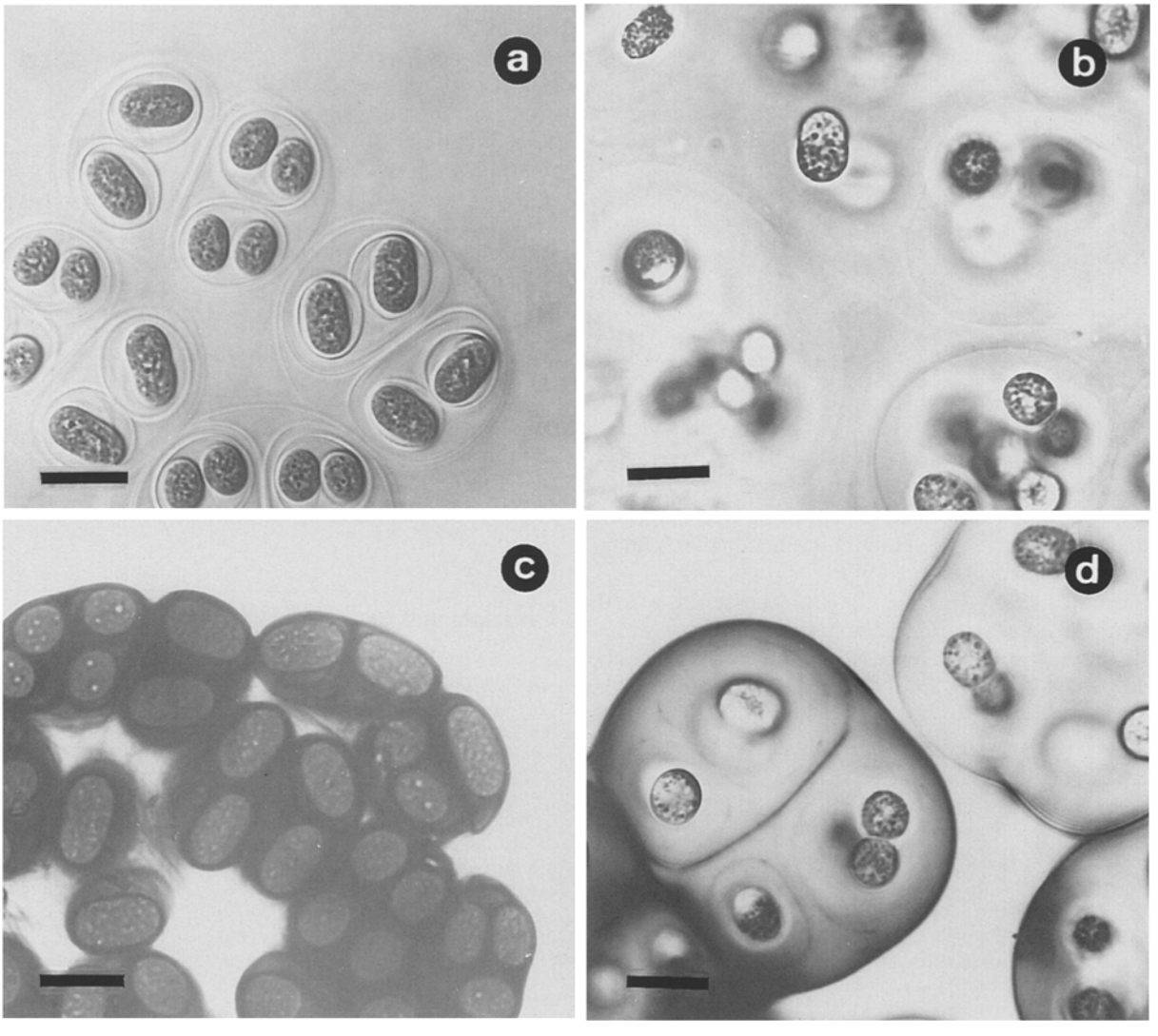

Fig. 1 Light micrographs of *Gloeothece* sp. PCC 6909 cells grown with sulfate (**a, c**) and without sulfate (**b, d**). To increase contrast, cells and sheath were stained with methylene blue (**c, d**). *Bars* 10 μm

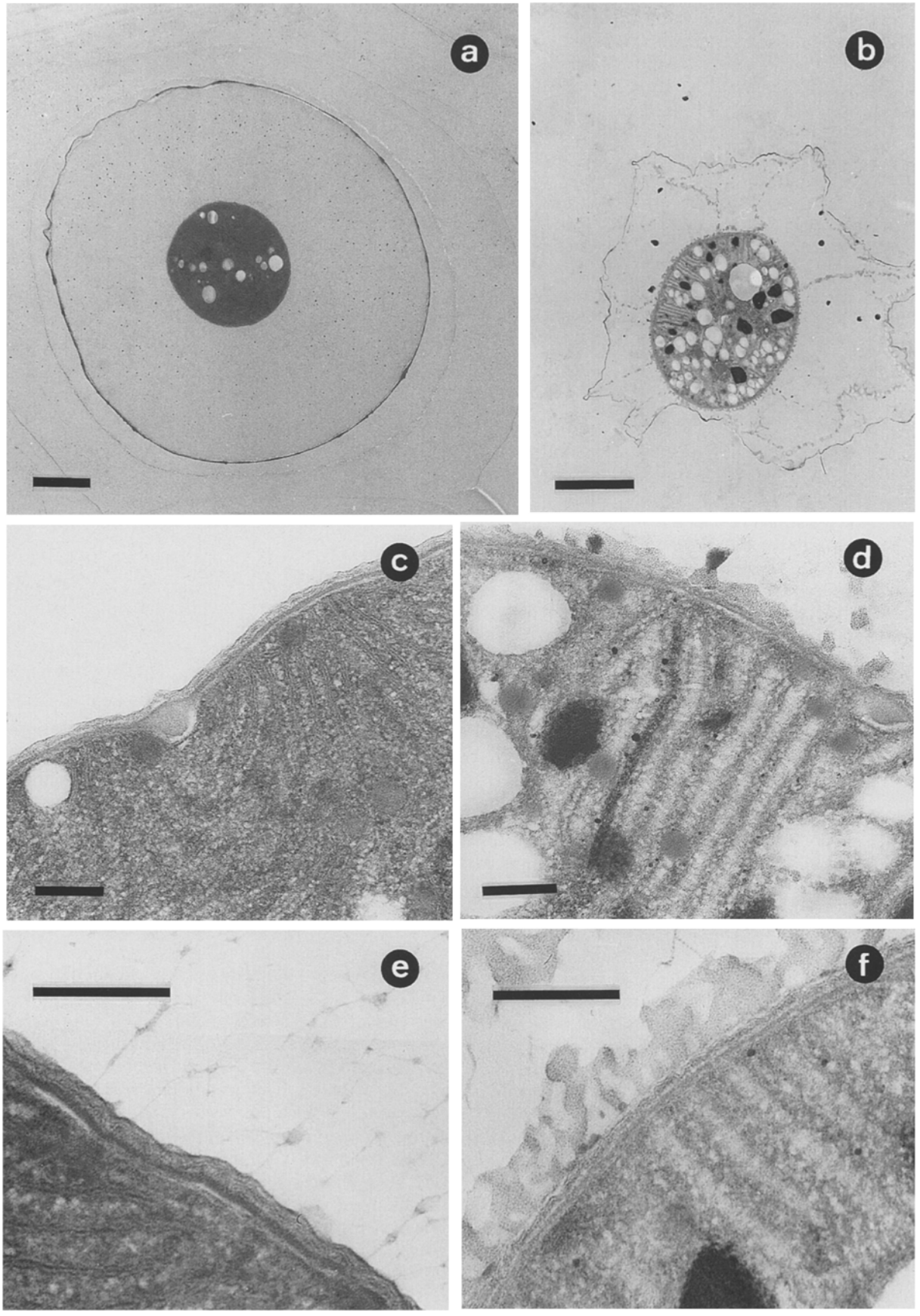

Fig. 2a–f TEM micrographs of *Gloeothece* sp. PCC 6909. **a** Cell grown with sulfate; **b** cell grown without sulfate, containing high number of cyanophycin (electron-dense bodies) and PHB (transparent spaces) granules; **c** detail of cell wall and thylakoids in a cell grown with sulfate; **d** detail of cell wall and thylakoids in a cell grown without sulfate; **e** sheath microfibrils in a cell grown with sulfate; **f** sheath microfibrils in a cell grown without sulfate. *Bars* 2 μm (**a, b**) and 0.2 μm (**c–f**)

the presence of sulfate were intense green and surrounded by a refringent and apparently compact sheath, divided in several concentric layers. This is a morphological characteristic commonly used in taxonomy. Cells were grouped in dense clusters with up to 8–16 cells, and sometimes aggregated further, forming macroscopically visible colonies. Such an arrangement can be explained as follows: when the cell divides, the sheath expands and surrounds the daughter cells, originating an external or secondary sheath that contains one or more pairs of cells. Cells grown without sulfate were yellowish with a granular cytoplasm and a larger and expanded sheath; however, the sheath was less refringent and apparently less compact, and frequently broken and dispersed, unable to englobe and hold together the daughter cells after division.

Cell wall and external layers

While the cell wall and plasmatic membrane of *Gloeothece* sp. PCC 6909 growing in the absence of sulfate did not appear to undergo any type of alteration, TEM showed marked differences in the ultrastructure of the sheath that surrounded the cells. When *Gloeothece* sp. PCC 6909 was grown with sulfate, a perfectly formed sheath was observed, structured in concentric layers and separated by electron-dense bands that surrounded the cells individually (Fig. 2a). The observed electron density, derived mainly from the lead citrate stain, suggests that acidic mucopolysaccharides were present. The sheath produced in the presence of sulfate had a fibrillar structure (Fig. 2e). The microfibrils were approximately radially oriented, more visible in the inner layers of the sheath. Cells grown without sulfate were surrounded by structureless sheaths (Fig. 2b, f). It was not possible to distinguish the organization in concentric layers, and randomly distributed microfibrils produced an amorphous, diffuse sheath. Sulfur deficiency caused the disappearance of the electron-dense concentric layers observed in cells grown with sulfate.

X-ray mapping of elemental distribution in both types of cell showed a marked accumulation of sulfur in the cells and in the sheaths, especially in the electron-dense concentric layers when *Gloeothece* sp. PCC 6909 was grown with sulfate (Fig. 3). In the absence of sulfate, only traces of sulfur were detected, and these traces were not arranged in layers. Although sulfur-starved cells obviously contained proteins, the amount of sulfur localized in these proteins was, in many cases, probably below the detection limit of the technique.

Cell inclusions

The variation in size and number of cell inclusions, depending on the sulfate availability, is also noteworthy (Fig. 2). Few cyanophycin granules were observed when the cells were grown with sulfate. In contrast, in sulfate-deficient cultures, the number of granules was much higher.

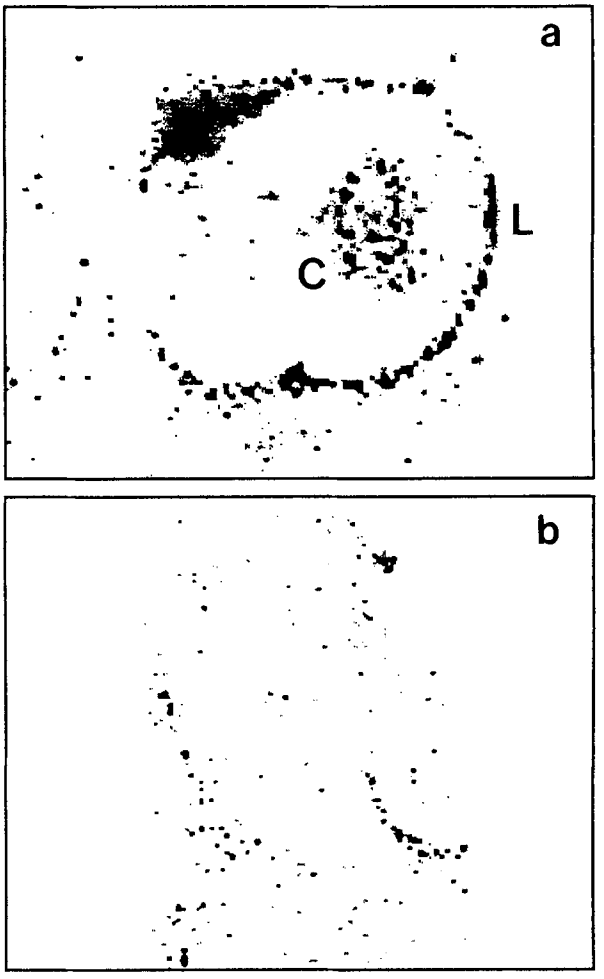

Fig. 3 Sulfur distribution in a STEM micrograph of a *Gloeothece* sp. PCC 6909 cell grown: **a** with sulfate; **b** without sulfate (*C* cell, *L* concentric dense layer in the sheath)

Sulfur starvation caused an increase of "storage granules," which appeared as transparent or slightly electron-dense spaces (Fig. 2d). In the presence of sulfate, the transparent spaces, although not very abundant, were distributed regularly in the cytoplasm, occupying a restricted volume of the cell. In cells grown without sulfate, there was an increment in average size and a considerable proliferation of such granules, which eventually occupied almost all the cytoplasm. These structures have been identified as polyphosphate and polyhydroxybutyrate (PHB) reservoirs and accumulate in *Gloeothece* sp. PCC 6909 during diazotrophic growth (Rippka et al. 1971). Stal (1992) reviewed the presence of polyhydroxyalkanoates (PHA) in cyanobacteria and studied their pattern of accumulation and mobilization. He has suggested that PHA granules in cyanobacteria can be easily confused with polyphosphate inclusions in studies based solely on electron microscopy, as can be seen from the scant number of reports on PHA in cyanobacteria. We applied analytical pyrolysis as a tool to prove chemically the possible presence of PHB in the whole cell as a result of sulfur starva-

tion in *Gloeothece* sp. PCC 6909. Standard PHB and sulfur-deficient cells were pyrolysed. The total ion current (TIC) chromatograms are shown in Fig. 4. The homopolymer of 3-hydroxybutanoic acid or PHB yielded two main pyrolysis products: 2-butenoic acid (monomer) and its dimer. These compounds were also identified in the pyrolysate of the whole cells, which unambiguously indicates the presence of PHB. However, in cells grown with sulfur, the typical pattern of pyrolysis products from PHB was not found, and only 2-butenoic acid was obtained. This indicates either that the precursor 3-hydroxybutanoic acid is present in the non-starved cells, but does not produce the polymer, or that if a polymer is produced, the structural units are different than those of PHB, and consequently than its pyrolysis pattern. The evidence of PHB by pyrolysis suggests that a major part of the transparent reserve spaces appearing under sulfur starvation can be attributed to PHB inclusions. However, the presence of polyphosphate granules cannot be ruled out, as diazotrophic *Gloeothece* sp. cultures are known to contain significant amounts of acid-soluble polyphosphate (Gallon et al. 1988).

Thylakoids

When *Gloeothece* sp. PCC 6909 was grown with sulfate, numerous convoluted thylakoids occupied a considerable fraction of the cytoplasm. They had the typical structure of a double-membrane with an electron-dense intrathylakoidal space and were disposed in parallel groups showing a radial or subradial arrangement in the peripheral regions of the cells. Glycogen granules were located among the thylakoids (Fig. 2c). The absence of sulfate caused most of the thylakoids to disappear, and those that remained were vacuolized, and it was difficult to distinguish the double-membrane structure. In this case, the cytoplasm had a granulose aspect, caused by the presence of a considerable quantity of glycogen granules. This ultrastructural modification was accompanied by bleaching of the cultures due to pigment degradation.

Cell recovery from sulfur starvation

In natural conditions, the availability of any particular nutrient varies from periods in which a limited amount (or none) is present to periods of sufficiency. Therefore, an experiment was carried out to determine the morphological and ultrastructural alterations during the process of cell recovery from sulfur starvation in *Gloeothece* sp. PCC 6909. Observations using light microscopy revealed that the addition of 1 g of $CaSO_4$ to sulfur-starved cultures resulted in an increased refringence of the sheaths surrounding the cells after 12 h. Cultures turned slightly green after day 2, although a blue-green color was not totally restored until day 4. After this lag period, cell division caused an increase in the number of cells present in the colonies, which often contained more than 16 cells.

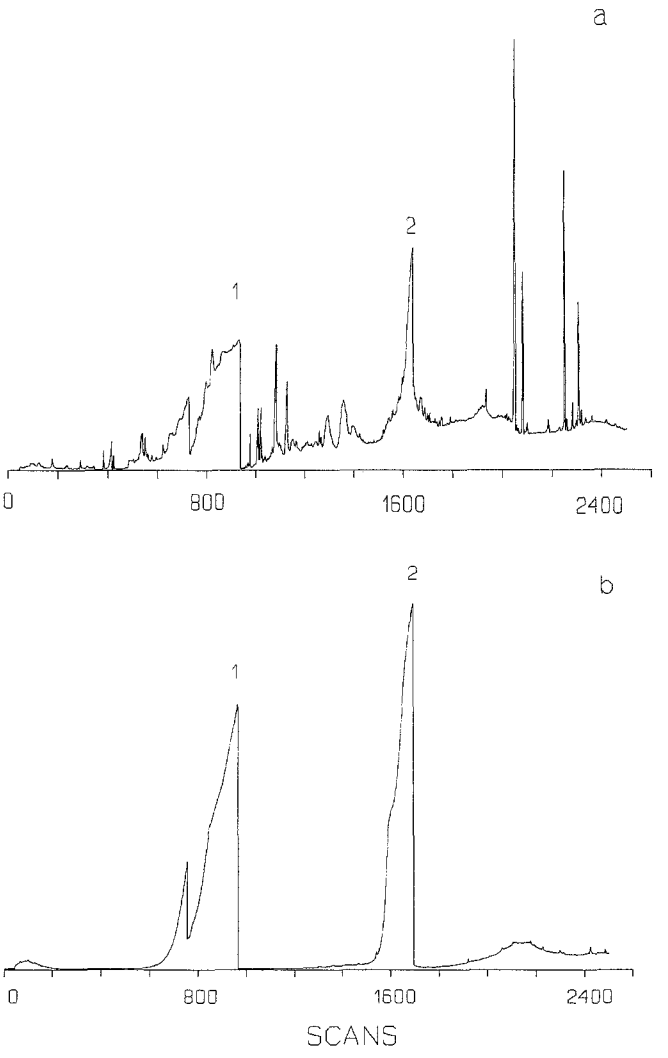

Fig. 4 Total ion current chromatogram of **a** *Gloeothece* sp. PCC 6909 biomass grown without sulfate and **b** polyhydroxybutyrate (PHB). *1* 2-Butenoic acid, *2* dimer

After 8 days, newly formed sheaths were observed, either surrounding individual cells or surrounding pairs of cells undergoing division. TEM showed that cells that had recovered from sulfur starvation still contained a significant number of PHB and cyanophycin granules in the cytoplasm, as did sulfur-starved cells (Fig. 5a, c). Ruthenium-red staining confirmed the observed alterations in sheath ultrastructure during sulfur starvation. While sulfur-starved cells were surrounded by a structureless sheath formed by abundant microfibrils (Fig. 5a), sheaths synthesized de novo with sufficient sulfate consisted of well-defined dense bands and a network of radially oriented microfibrils that surrounded individual cells (Fig. 5c). Recovery from sulfur starvation also resulted in the reorganization of the thylakoids and the disappearance of the abundant glycogen granules that were present in starved cells (Fig. 5b, d).

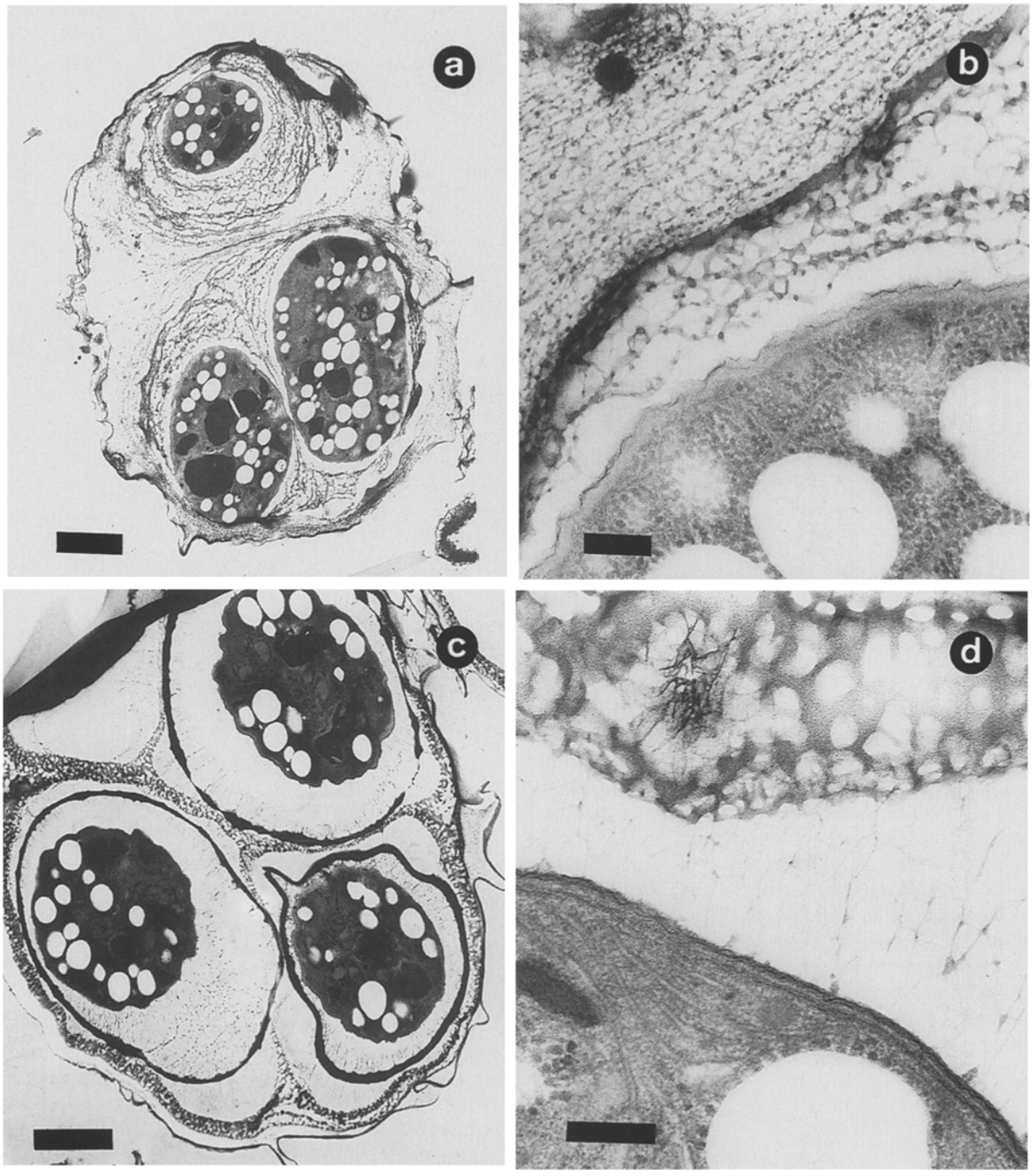

Fig. 5a–d TEM micrographs of ruthenium-red-stained *Gloeothece* sp. PCC 6909 cells showing cell recovery from sulfur starvation. Micrographs show **a** sulfur-starved cells and **c** recovered cells from the same culture 8 days after sulfate addition. Details of sheath, cell wall, and thylakoids from **b** sulfur-starved and **d** recovered cells. *Bars* 2 μm (**a, c**) and 0.2 μm (**b, d**)

Discussion

The sulfate-bonding capacity of the sheath in *Gloeothece* sp. PCC 6909 causes a particularly high demand for this anion. This makes sulfur nutrition a relevant aspect in the physiology of this cyanobacterium, which had not been covered by earlier ultrastructural studies.

The usual bleached aspect of *Gloeothece* sp. PCC 6909 cultures maintained with N_2 as a sole source of nitrogen has been formerly attributed to nitrogen depletion because of malfunctioning of nitrogenase enzyme under oxic conditions. Ortega-Calvo and Stal (1994) have proven that such bleaching was caused by sulfur depletion due to massive incorporation of all the sulfate initially present in the culture medium into the sheath. The aim of our study was not to confirm previous findings, but to characterize ultrastructural changes caused by sulfur starvation in this

organism. We showed that, when deprived of sulfur, bleached cells contain a disintegrated photosynthetic apparatus and accumulations of different kinds of reserve material. Such characteristics were absent in sulfate-sufficient diazotrophic cultures. The inability to obtain an adequate supply of sulfur for protein synthesis from the culture medium and therefore for balanced growth, induced in *Gloeothece* sp. PCC 6909 the immobilization of fixed carbon in the form of glycogen, PHB, and cyanophycin. The latter also acts as a reserve for fixed nitrogen that cannot be used for protein synthesis. Under such conditions, phycobiliproteins are degraded and used as emergency source of sulfur. The observed reduction in the photosynthetic membranes is possibly a means of preventing their photooxidative damage caused by a reduced utilization of light energy for photosynthesis.

Cell recovery from sulfur starvation required a short lag period, after which thylakoids were rapidly reorganized, glycogen granules disappeared as a result of the use of this storage material, cell division resumed, and a new, well-organized sheath was formed. PHB and cyanophycin granules were, however, still visible.

Ultrastructural changes due to sulfur starvation have also been observed in other cyanobacteria. For example, in sulfur-starved cells of *Synechococcus* 6301, where there is a fast degradation of *c*-phycocyanin and the chlorophyll content is drastically decreased, the thylakoid system is reduced, and glycogen and PHB granules occupy a large portion of the cell volume (Schmidt et al. 1982; Wanner et al. 1986). Cells of *Synechococcus* sp., when cultured at low-sulfate concentrations, contain an increased number of polyphosphate granules (Lawry and Jensen 1979). Sulfur starvation has also been found to induce cyanophycin accumulation in *Plectonema boryanum*, *Anabaena variabilis*, *Nostoc muscorum*, and *Phormidium luridum* (Lawry and Simon 1982), and *Aphanocapsa* sp. (Allen et al. 1980).

This investigation included a detailed examination of sheath ultrastructure and sulfur distribution within the sheath, which, to our knowledge, has not been reported previously in other ultrastructural studies of sulfur-starved cyanobacteria. Ruthenium-red staining of cells for TEM clearly evidenced that, in *Gloeothece* sp. PCC 6909, sulfate plays an essential role in exopolysaccharide microfibrillar organization and consequently in sheath organization. Observations of some cyanobacteria indicate that characteristics of the external layers may vary with age and culture conditions (Tischer and Davis 1971; Tease and Walker 1987). In *Gloeothece* sp. PCC 6909, the thickness and structure of the sheath are influenced by sulfate availability.

We conclude that *Gloeothece* sp. PCC 6909 reacts to sulfur starvation by notable changes in its ultrastructure related to alteration in the overall physiological processes, including sheath synthesis, nitrogen metabolism, and photosynthesis. Most of these alterations can be reversed by the addition of sulfate to the culture medium.

Many of the previous physiological and biochemical studies on this strain have been probably made with sulfur-deficient cells. The questions arise as to whether these studies can be considered representative and whether further studies must be carried out after supplementation of this nutrient deficiency.

Acknowledgements This study was supported by the C.E.C. (project EV5V-CT92-0112). The facilities and the kind assistance of the staff of the Electron Microscopy Service, University of Barcelona, and the technical assistance of B. Hermosin in analytical pyrolysis are acknowledged.

References

Allen MN, Hutchinson F, Weathers P (1980) Cyanophycin polypeptide formation and degradation in the cyanobacterium *Aphanocapsa* 6308. J Bacteriol 141:687–693

Gallon JR, Perry SM, Rajab TMA, Flayeh KAM, Yunes JS, Chaplin AE (1988) Metabolic changes associated with the diurnal pattern of N_2 fixation in *Gloeothece*. J Gen Microbiol 134: 3079–3087

Hernandez-Marine M (1992) A simple way to encapsulate small samples for processing for TEM. J Microsc 168:203-206

Lange W (1976) Speculation on a possible essential function of the gelatinous sheath of blue-green algae. Can J Microbiol 22: 1181–1185

Lawry NH, Jensen TE (1979) Deposition of condensed phosphate as an effect of varying sulfur deficiency in the cyanobacterium *Synechococcus* sp. Arch Microbiol 120:1–7

Lawry NH, Simon RD (1982) The normal and induced occurrence of cyanophycin inclusion bodies in several blue-green algae. J Phycol 18:391–399

Ortega-Calvo JJ, Stal LJ (1994) Sulphate-limited growth in the N_2-fixing unicellular cyanobacterium *Gloeothece* (Nageli) sp. PCC 6909. New Phytol 128:273–281

Rippka R, Neilson A, Kunisawa R, Cohen-Bazire G (1971) Nitrogen fixation by unicellular blue-green algae. Arch Microbiol 76:341-348

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61

Schmidt A, Erdle I, Köst P (1982) Changes of *c*-phycocyanin in *Synechococcus* 6301 in relation to growth on various sulfur compounds. Z Naturforsch [C] 37:870–876

Stal LJ (1992) Poly(hydroxyalkanoate) in cyanobacteria: an overview. FEMS Microbiol Rev 103:169–180

Tease B, Walker RW (1987) Comparative composition of the sheath of the cyanobacterium *Gloeothece* ATCC 27152 cultured with and without combined nitrogen. J Gen Microbiol 133:331–339

Tease B, Jürgens UJ, Golecki JR, Heinrich, UR, Rippka R, Weckesser J (1991) Fine-structural and chemical analyses on inner and outer sheath of the cyanobacterium *Gloeothece* sp. PCC 6909. Antonie Van Leeuwenhoek 59:27–34

Tischer RG, Davis EB (1971) The effect of various nitrogen sources upon the production of extracellular polysaccharide by the blue green alga *Anabaena flos-aquae* A37. J Exp Botany 22:546–551

Vaara T (1982) The outermost surface structures in chroococcacean cyanobacteria. Can J Microbiol 28:929–941

Wanner G, Henkelmann G, Schmidt A, Köst HP (1986) Nitrogen and sulfur starvation of the cyanobacterium *Synechococcus* 6301. An ultrastructural, morphometrical and biochemical comparison. Z Naturforsch [C] 41:741–750

Weckesser J, Broll C, Adhikary SP, Jürgens UJ (1987) 2-*O*-Methyl-D-xylose-containing sheath in the cyanobacterium *Gloeothece* sp. PCC 6501. Arch Microbiol 147:300–303