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Exopolysaccharide production by a unicellular cyanobacterium isolated from a hypersaline habitat

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Abstract

The unicellular cyanobacterial strain 16Som2, isolated from a Somaliland saltpan and identified as *Cyanothece* sp., is characterized by cells surrounded by a thick polysaccharidic capsule, the external part of which dissolves into the medium during growth, causing a progressive increase in culture viscosity. In spite of this, the thickness of the capsule remained almost constant under all the culture conditions tested, demonstrating that the processes of its synthesis and solubilization occurred at a similar rate. The synthesis of carbohydrates was neither enhanced by increasing salinity (sea-water enriched with NaCl in the range 0 to 2.0 M) nor by Mg^{2+}, K^+ or Ca^{2+} deficiencies. In contrast, N-limitation and, to a lesser extent, P-limitation induced a significant enhancement of carbohydrate synthesis; in particular, N-deficiency stimulated the synthesis of all the carbohydrate fractions (intracellular, capsular and soluble). The soluble polysaccharide, separated from the culture medium and hydrolyzed with 2N trifluoroacetic acid, showed a sugar composition consisting of glucuronic acid: galacturonic acid: galactose: glucose: mannose: xylose: fucose in a molar ratio of 1: 2: 2.4: 6.8: 4.8: 2.9: 1.6. *Cyanothece* sp. culture subjected to nitrogen starvation synthesized polysaccharide with a mean productivity of 115 mg (EPS) $l^{-1}d^{-1}$, for the polymer solubilized into the medium, and of 15 mg (CPS) $l^{-1}d^{-1}$ for the capsular polysaccharide.

Abbreviations: CPS = capsular polysaccharide, EPS = exocellular polysaccharide, PAR = photosynthetic active radiation

Introduction

The increased demand for natural biopolymers for various industrial applications (Lewis *et al.*, 1988; Linton *et al.*, 1991) has led in recent years to a renewed interest in exopolysaccharide production by cyanobacteria. Many studies are now available, on both unicellular and filamentous cyanobacteria of freshwater or marine origin, concerning either the chemical composition of the polysaccharides and the influence of different growth conditions on EPS production (Painter, 1983; Panoff *et al.*, 1988; Phlips *et al.*, 1989; Vincenzini *et al.*, 1990a, b; De Philippis *et al.*, 1991),

and the rheological properties of both whole cyanobacterial cultures (Lapasin *et al.*, 1992) and aqueous solutions of purified polysaccharides (Navarini *et al.*, 1990). However, although promising results were obtained, studies were limited to a few cyanobacterial strains and additional work is necessary to evaluate the potential of cyanobacteria as a new source of these biopolymers. The marked differences in monosaccharidic composition so far observed in cyanobacterial polysaccharides (Vincenzini *et al.*, 1990a) suggest the possibility of finding new strains that produce polymers possessing chemical or rheological properties as to make them suitable for new applications. In particular, many unicellular cyanobacteria living in marine and hypersaline environments (saltworks, lakes, ponds, basins etc) are characterized by cells surrounded by a mucilaginous envelope (Borowitzka, 1981; Campbell & Golubic, 1985), so that it is very likely that they may include strains capable of releasing large amounts of exopolysaccharide into the culture medium. On the other hand, the utilization of EPS-producing cyanobacterial strains able to grow on hypersaline media suggests the possibility of their being cultivated in marginal lands, thus allowing the utilization of underexploited resources for the production of valuable chemicals, as suggested by Aller (1982).

We isolated, from a Somaliland saltpan, a unicellular cyanobacterial strain that we identified as *Cyanothece* sp. This strain, maintained in axenic culture in our laboratory for several years, is characterized by cells surrounded by a thick mucilaginous capsule; the external part of this capsule, during growth, dissolves into the medium, so causing a progressive increase in the viscosity of the culture.

In order to investigate the conditions that can enhance the production of the exocellular polysaccharide by *Cyanothece*, we assayed the effects of salinity and of those nutritional deficiencies known to increase the synthesis of carbohydrates in cyanobacteria and in microalgae (Kroen & Rayburn, 1981; Arad *et al.*, 1988; De Philippis *et al.*, 1991).

Materials and methods

Organism and growth conditions

The strain 16Som2 was isolated from a sample of microbial mat collected in a saltpan near Getzira (Somaliland). It was obtained in pure culture by a procedure based on equilibrium density gradient centrifugation. The cells, washed in 2M NaCl solution containing 1% (w/v) SDS to remove the capsule, were suspended in a 1:1 (v/v) solution of Percoll (Pharmacia, Sweden) and of 2M NaCl and centrifuged at 75000 × *g* for 15 min at 10 °C. The cyanobacterial cells became more concentrated and apparently free from contaminating bacteria at the top of the gradient; aliquots of this cyanobacterial suspension were plated in enriched seawater medium (see below) solidified by adding 0.9% (w/v) agar, from which the strain was isolated in pure culture. The organism was grown axenically in 500-ml Erlenmeyer flasks, containing 200 ml of sea-water medium supplemented as follows (g l^{-1}): $NaNO_3$, 1.5; Na_2HPO_4, 0.04; $NaHCO_3$, 0.1; NaCl, 28; ferric-ammonium citrate, 0.006; citric acid, 0.006; Na_2 EDTA, 0.001 and 1 ml l^{-1} of trace metal solution A_5 + Co (Rippka *et al.*, 1979). The cultures were incubated in an orbital shaker (Psycrotherm mod. 627, New Brunswick Scientific Co, USA) at 30 °C under continuous illumination provided by cool white fluorescent tubes giving 80 μmol photon m^{-2} s^{-1} (PAR) at the flask surface. The growth chamber was continuously flushed with a mixture of air–CO_2 (95%–5%, v/v). Owing to the interference of nitrate in carbohydrate analysis, in all the experiments $NaNO_3$ was replaced by NH_4Cl; in order to avoid the toxic effects of high NH_4^+ concentrations, NH_4Cl 2.8 mM was added every two to five days, according to the growth rate of the cultures and to the residual concentration of ammonium in the medium. The pH was maintained in the range 7.5–7.8 by adding, when required, sterile Na_2CO_3. The effect of different salinities on growth and on carbohydrate synthesis was examined in batch cultures using enriched sea-water medium (modified by substitution of $NaNO_3$ with NH_4Cl, as above de-

scribed) with the addition of NaCl at concentrations ranging between 0.5 and 2M. The effects of the shortage of some nutriens (K^+, Ca^{2+}, Mg^{2+}, HPO_4^{2-} and NH_4^+) were examined by growing the strain on ASN-III medium (Rippka *et al.*, 1979) modified by adding 0.5 M NaCl and NH_4Cl 2.8 mM, instead of $NaNO_3$. Nitrogen starvation was obtained by omitting NH_4Cl periodical reintegration; depletion of other elements was obtained by growing *Cyanothece* cells for 30 days in ASN-III medium free of the specific element, diluting the culture every week with fresh medium to the initial cell concentration. Experiments aimed at evaluating the amount of the different carbohydrate fractions both in standard (2.8 mM NH_4Cl periodically added) and in N-limited growth conditions (2.8 mM NH_4Cl supplied only once at the beginning of the experiment) were carried out in batch in 2000 ml Fernbach flasks containing 800 ml of culture on enriched seawater medium devoid of nitrate.

Polysaccharide separation

For exopolysaccharide separation, cells were removed from the growth medium by centrifugation (14000 × **g**, 10 min at 10 °C). Capsular polysaccharide was solubilized by stirring capsulated cells in 1.5% (w/v) NaCl solution (30 min, 60 °C) and by centrifuging at 48000 × **g** (20 min at 10 °C). Soluble and capsular polysaccharides were obtained from the two supernatants according to the procedure described by Vincenzini *et al.* (1990b).

Analytical procedures

Cyanothece 16Som2 cells were observed, using bright-field illumination, with a Polyvar photomicroscope (Reichert-Jung, Austria) before and after staining with Indian ink. Cell growth was estimated following the increase in protein content of the cultures, determined according to the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (Sigma Chemical Co, USA) as a standard. Total carbohydrates were determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956), using glucose as a standard. The $N-NH_4^+$ content of the medium was determined by the phenol-hypochlorite method (Solorzano, 1969), after cell removal by filtration through glass fiber discs. Nitrogenase activity, both in aerobic and in anaerobic conditions, was assayed by the acetylene reduction technique (Turner & Gibson, 1980). For the detection of anaerobic nitrogen fixation, the procedure described by Rippka & Waterbury (1977) was adopted. The monosaccharidic composition of the EPS was determined after hydrolysis in 2N trifluoroacetic acid at 120 °C for 10 or 60 min. Different lengths of the hydrolysis period were required to optimize the yield of single sugars. The hydrolysates were analyzed by HPLC according to the method described by Vincenzini *et al.* (1990b). Corrective factors for uronic acid quantification were obtained by means of HPLC analyses of standard solutions after treatment with 2N trifluoroacetic acid; the quantitative results obtained with these corrective factors fitted well the concentration of total uronic acids determined by the carbazole method (Galambos, 1967). Total nitrogen content of EPS samples was determined by the Kjeldahl method. Infrared spectra of EPS preparations were obtained as previously described (Vincenzini *et al.*, 1990b).

Results

The unicellular cyanobacterial strain 16Som2 showed widely ellipsoidal motile cells, 5–11 μm wide, 6–12 μm long, having a pale blue-green cell content, finely granulated or keritomized. Cell division usually occurred on one plane perpendicularly to the longitudinal axis (Fig. 1a), but in aged cultures asymmetrical crosswise divisions were sometimes observed (Fig. 1b). By Indian ink staining, cells appeared surrounded by a mucilaginous capsule (Fig. 1c), the thickness of which (about 7.8–9 μm) remains almost constant in different culture conditions: only in aged cultures (over two months old) was slight reduction gen-

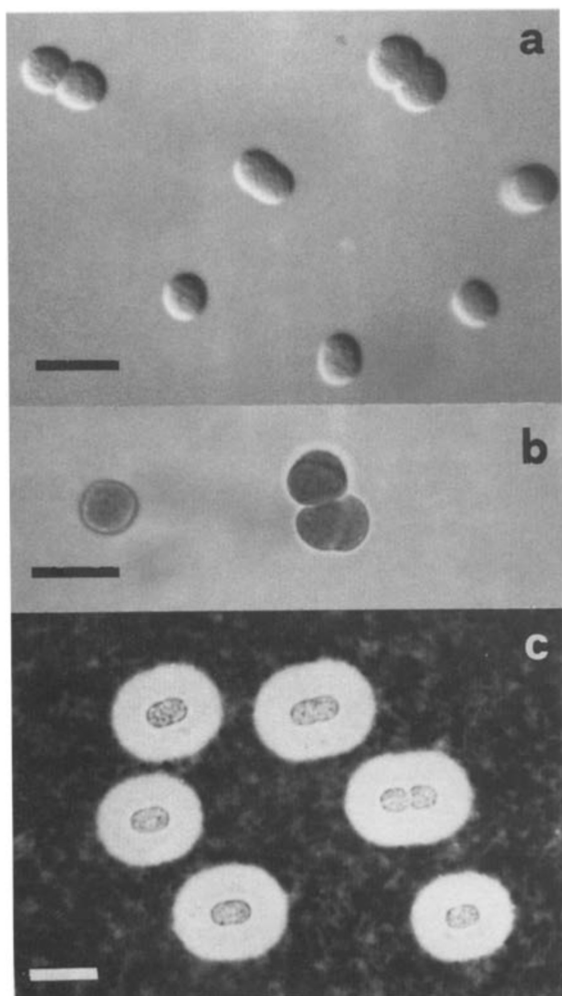

Fig. 1. Photomicrographs of *Cyanothece* sp. strain 16Som2: (a) cells from an actively growing culture (Nomarski differential interference contrast); (b) cells from a two months old culture showing asymmetrical crosswise division (bright-field illumination); (c) negatively stained cells. *Bar* = 15 μm.

erally observed. The cyanobacterium did not exhibit any nitrogenase activity either in aerobic or in anaerobic conditions. Owing to its morphological characteristics, the strain was identified as *Cyanothece* sp. (*sensu* Waterbury & Rippka, 1979).

The analysis of the biochemical composition of *Cyanothece* cells grown for 7 days on media containing different NaCl concentration (Fig. 2) showed that the increase of salinity only slightly affected the final cell yield, without enhancing car-

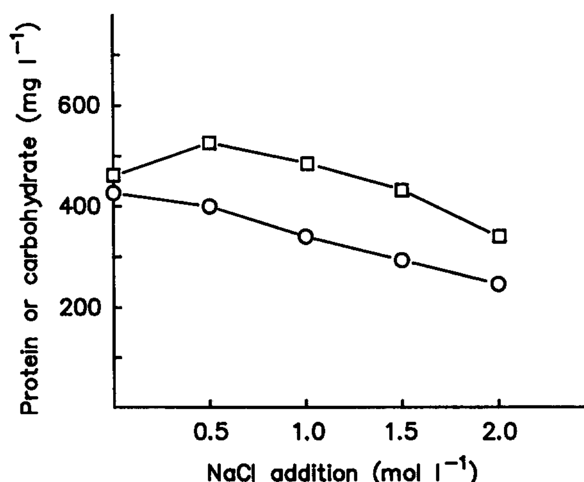

Fig. 2. Crude proteins (□) and total carbohydrates (○) in *Cyanothece* 16Som2 batch cultures after 7 days of growth on artificial sea-water medium enriched with different NaCl amounts. (At initial time, crude proteins = 50 mg l^{-1} and total carbohydrates = 37 mg l^{-1}).

bohydrate synthesis. However, the strain showed the capability to grow well in a wide range of salt concentrations, as was expected for a microorganism whose natural habitat is characterized by wide fluctuations of salinity.

The effects on growth and on total carbohydrate synthesis induced by various nutritional deficiencies in *Cyanothece* sp. batch cultures are reported in Table 1: after 6 days of growth, the highest concentration of total carbohydrates as well as the highest carbohydrate to protein ratio were observed in N-limited culture. Among the other nutritional deficiencies tested, only P-limitation induced a significant enhancement of carbohydrate synthesis, but to a lesser extent than nitrogen deficiency. A subsequent experiment, aimed at quantifying the contribution of the different carbohydrate fractions to the enhancement of sugar synthesis observed under N-limitation, showed that, as soon as NH_4Cl was exhausted, cell growth ceased (Fig. 3) and, at the same time, the specific rate of carbohydrate synthesis suddenly increased from about 0.16 to 0.67 mg of total carbohydrates per mg of crude protein per day. In the subsequent period, the specific rate of carbohydrate synthesis progressively decreased both in N-limited and in control culture, always

Table 1. Effect of different nutritional deficiencies on protein and carbohydrate synthesis in *Cyanothece* 16Som2 batch cultures after 6 days of growth. (At initial time, crude proteins = 130 mg l^{-1} and total carbohydrates = 98 mg l^{-1}). Nutrient starvations were obtained as described in Materials and methods.

	Crude proteins (mg l^{-1})	Carbohydrates (mg l^{-1})	Carbohydrates/Proteins ratio
Control	680	500	0.74
Mg^{2+} shortage	225	238	1.06
K^+ shortage	435	506	1.16
Ca^{2+} shortage	524	379	0.72
HPO_4^{2-} shortage	413	710	1.72
N-limitation	390	1075	2.76

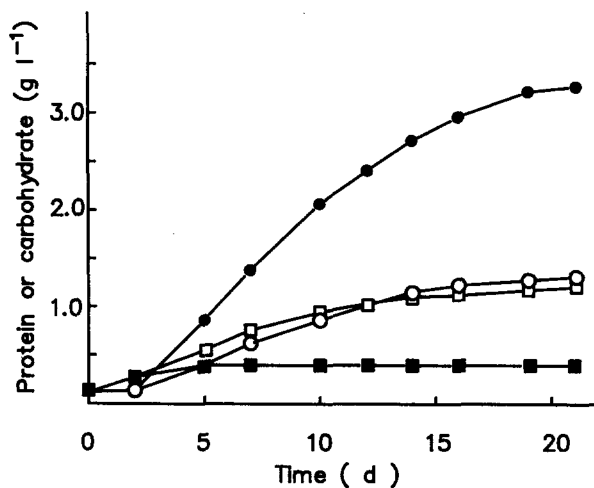

Fig. 3. Time course of crude proteins (■,□) and total carbohydrates (●,○) in *Cyanothece* 16Som2 batch cultures with (closed symbols) or without (open symbols) nitrogen limitation.

remaining higher however under nitrogen deficiency. As a consequence, after 21 days N-starved culture showed a significantly higher concentration of all the carbohydrate fractions (intracellular, capsular and soluble): in particular, the amount of EPS solubilized into the medium was 4 times higher in N-limited culture than in the control (Table 2).

Cyanothece culture subjected to nitrogen starvation synthesized the polysaccharide with a mean productivity, calculated at the end of culture period, of 115 mg (EPS) l^{-1} d^{-1} for the polymer released into the culture medium and of 15 mg (CPS) l^{-1} d^{-1} for the capsular polysaccharide. These figures are quite comparable with those reported for other photosynthetic microorganisms (Arad, 1988; Thepenier *et al.*, 1988; Vincenzini *et al.*, 1990b).

Aqueous solutions of EPS showed a positive reaction to carbazole, thus demonstrating that the polymer contains uronic acid(s), which accounted for 15.0% of crude EPS weight. Polymer samples showed a nitrogen content of about 1.4%, whereas IR spectra excluded the presence of sulphate residues, the two typical bands at 1240 and 820 cm^{-1} being absent, due respectively to S–O and C–O–S stretching vibrations (Lloyd & Dodgson, 1959). Samples of the exopolysaccha-

Table 2. Distribution of different carbohydrate fractions in *Cyanothece* 16Som2 batch cultures grown (21 days) with or without N-limitation.

	Intracellular carbohydrates (mg l^{-1})	Capsular polysaccharide (mg l^{-1})	Soluble polysaccharide (mg l^{-1})
Nitrogen limitation	475	330	2420
Control culture	420	170	610

ride, hydrolyzed with 2N trifluoroacetic acid and analyzed by HPLC, showed a sugar composition consisting of two acidic sugars (glucuronic and galacturonic acid), and five neutral sugars (galactose, glucose, mannose, xylose, fucose). The molar ratio among the different monosaccharides was calculated by comparing quantitative results of colorimetric and HPLC analyses, uronic acids being easily degradable during the hydrolisis, as was previously reported for other bacterial polysaccharides (Cesaro *et al.*, 1990). The results, obtained by using corrective factors for uronic acids, suggested a molar ratio of 1: 2: 2.4: 6.8: 4.8: 2.9: 1.6 among glucuronic acid: galacturonic acid: galactose: glucose: mannose: xylose: fucose. From these results, it is evident that the polysaccharide produced by the *Cyanothece* strain 16Som2 does not have a unusual composition: indeed, the monosaccharides present in this polymer are known to constitute, in different arrangements and proportions, many of the EPS produced by cyanobacteria (Vincenzini *et al.*, 1990a). The polymer also showed gelling properties: the addition of drops of EPS aqueous solution (1% w/v) to 0.05M $FeCl_3$ or $CuCl_2$ solution led to the formation of gel beads, but only those formed in the presence of ferric ions showed a stable shape for over two weeks, those formed in copper solution dissolving easily in less than 60 min.

Discussion

Cyanothece sp. strain 16Som2 appears similar, as regards its morphological characteristics, to the coccoid slime-producing cyanobacteria which are frequently dominant in hypersaline habitats (Brock, 1976; Campbell & Golubic, 1985; Margheri *et al.*, 1987) and, in particular, it seems to be very close to the coccoid cyanobacterium *Cyanothece halobia* isolated from Hellenic heliothermal saltworks (Roussomoustakaki & Anagnostidis, 1991).

The almost constant capsule thickness observed in *Cyanothece* 16Som2 throughout the growth period seems to demonstrate that the processes of EPS synthesis and of EPS release occur at a comparable rate, as it was observed in *Cyanospira capsulata* batch cultures (Vincenzini *et al.*, 1990a, b), but in contrast with the behaviour shown by the red algae *Porphyridium* and *Rhodella*, whose capsule thickness increased with cell age (Arad *et al.*, 1988; Dubinsky *et al.*, 1990). Since the thickness of the capsule surrounding the cells did not significantly change under any of the growth conditions tested, it is possible to conclude that it is a stable cell structure of this cyanobacterium.

The behaviour observed in the culture subjected to N-starvation points out that, under this condition, the biosynthetic activities of *Cyanothece* cells are directed preferentially toward carbohydrate synthesis, being hindered in protein formation by the lack of nitrogen source; in this respect strain 16Som2 behaves like the EPS-producing microalgae *Chlamydomonas mexicana*, *Porphyridium* spp. and *Rhodella reticulata* (Kroen & Rayburn, 1984; Arad, 1988; Arad *et al.*, 1992), but differently from the other cyanobacteria so far investigated. Indeed, for *Synechocystis* PCC 6803 and PCC 6714 no influence of nitrogen limitation on the EPS production was reported (Panoff *et al.*, 1988). It is worth noting that in N-limited cultures of phototrophic microorganisms the amount of energy available for single cells decreases only slightly during the cultivation period. In fact, under this condition, cell division is inhibited and the progressive decrease of light availability due to the increase of shading among cells typical of batch cultures does not occur, thereby allowing cells to maintain a fairly high photosynthetic activity, mainly directed toward carbohydrate synthesis. Such a condition, characterized by the absence of growth along with the persistence of some specific biosynthetic activities, has been compared by Arad (1988) to the typical state of immobilized cell systems.

From the results reported above, it is possible to draw some conclusions:

(i) in *Cyanothece* strain 16Som2 the synthesized EPS does not seem to have the function of chelating agent since the production of the

polymer is affected more by N-limitation rather than by the lack of some essential cations like K^+, Ca^{2+} and Mg^{2+};

(ii) EPS productivity showed by the culture run under N-limitation makes the strain quite promising as photosynthetic EPS-producer;

(iii) the capability of this cyanobacterium to grow at high osmotic pressures is of particular interest giving the possibility of utilizing waters of various salinities as culture medium.

Only further investigations on the rheological properties of the polysaccharide and on the capability of the microorganism to produce a polymer with reproducible characteristics will lead to a full evaluation of the potential of *Cyanothece* 16Som2 as polysaccharide producer.

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