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Chemical composition and production of exopolysaccharides from representative members of heterocystous and non-heterocystous cyanobacteria

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Dedicated to the memory of Professor Giacomino Randazzo

Abstract

Exopolysaccharide (EPS) production and chemical composition of heterocystous and non-heterocystous cyanobacteria of subgroups 3, 4, and 5 together with four new species isolated from Pantelleria (Italy) hard sands and from a lake in the proximity of Edmonson Point in Antarctica were studied. The yield of extracellular soluble polysaccharides (RPS) and slime layers (CPS) showed a significant variation from one strain to another; generally RPS were more abundant than CPS with the exception of *Anabaena torulosa* and *Scytonema hoffmanni*. *Phormidium* sp. was the best producer of EPS. Among heterocystous cyanobacteria belonging to *Nostocaceae* the new species *Anabaena* WSAF and *A. torulosa* were found to produce the highest level of EPS. Among heterocystous cyanobacteria other than *Nostocaceae*, the best producer of EPS was *Chlorogloeopsis* sp. 6912. Four species were chosen for studying the effects of growth conditions, nutritional, physical and chemical, on total EPS formed. Glucose and galactose were the neutral sugars widely present in the species examined, although other sugars such as mannose and xylose were plentiful in some species. Amino-sugar, as glucosamine, and uronic acids were also found in EPS of some cyanobacteria. Finally the inhibition of avarol, a sesquiterpene hydroquinone, toxicity on *Artemia salina* by EPS of some species was analysed.

Keywords: Cyanobacteria; Exopolysaccharide; Capsular polysaccharide; Released polysaccharide; Exopolysaccharide composition; Biological activity

1. Introduction

Increasing attention has been received by the exopolysaccharides produced in large quantities by a wide range of cyanobacteria due to their commercial applications as industrial gums and to their participation in pathogenic and symbiotic processes in plants and animals and the general interactions between microorganisms and their environment (Morvan, Gloaguen, Vebret, Joset & Hoffman, 1997; De Philippis, Sili, Tassinato, Vincenzini & Materassi, 1991; Gloaguen, Morvan & Hoffman, 1995; Pulz & Koehler, 1994).

Little data are available in literature regarding the polysaccharides from the capsule and slime of cyanobacteria; such polysaccharides had been extracted via a procedure which may have isolated slime and /or capsule material too (Bertocchi, Navarini, Cesàro & Anastasio, 1990).

Extracellular carbohydrate polymer (EPS) production has been reported (e.g.) for *Anabaena cylindrica* (Bishop, Adams & Hughes, 1954; Lama et al.,

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Table 1
Production of released polysaccharides (RPS) and slime polysaccharide (CPS) from tested cyanobacteria

Cyanobacteria	CPS μg g^{-1} (cell fr. wt)	RPS μg l^{-1} (cell fr. wt)	Wet cells g l^{-1} (culture medium)
Non-heterocystous Subgroup 3 oscillatoriales			
Spirulina sp.	650	9000	13.0
Oscillatoria sp.	573	1500	3.5
Phormidium sp.	512	29000	1.6
Antarctic sp. 1	50	1900	1.2
Antarctic sp. 2	455	1000	1.5
Heterocystous Subgroup 4 Nostocaceae			
Anabaena torulosa	9100	3700	2.0
Anabaena sphaerica	394	1000	2.0
Anabaena WSAF	100	55200	4.5
Anabaena sp. 7120	150	20	1.3
Anabaena variabilis	90	3500	3.9
Nostoc linckia	720	15	1.0
Scytonemataceae			
Scytonema hofmanni	4400	4000	6.3
Tolypothrix tenuis	15	7400	4.0
Rivulariaceae			
Microchaete sp. 7126	1500	nd	4.0
Subgroup 5 Stygonematales			
Fischerella muscicola	800	1500	1.9
Chlorogloeopsis sp. 6912	1000	30000	3.0

1996), *Nostoc* species (Flaibani, Olsen & Painter, 1989; Hough, Jones & Wadman, 1952; Mehta & Vaidya, 1978; Moore & Tischer, 1964), *Palmella mucosa* (Tischer & Moore, 1964), and *Spirulina* species (Filali Mouhim, Cornet, Fontaine, Fournet & Dubertret, 1993; Tseng & Zhao, 1994). Almost all of the common monosaccharide units have been identified in these polymers (Gloaguen et al., 1995). The type and the amount of polysaccharide production depend on the species employed and cultivation conditions (Moore & Tischer, 1964; Sangar & Dugan, 1972). Few data exist about nutritional factors which may influence EPS production (De Philippis et al., 1991). It must be noticed that the sugar composition may slightly vary, both qualitatively and quantitatively, especially with the age of the culture (Gloaguen et al., 1995).

In this paper we report a comparative study of different species of cyanobacteria for the production of polysaccharides. We describe the isolation and chemical composition both of macromolecular material from the slime layer (capsular polysaccharides, CPS) of the cyanobacteria and of extracellular soluble polysaccharides (released polysaccharides, RPS) from their growth media. The influence of some physical and nutritional factors to optimize the production of the EPS as well as their possible pharmaceutical applications were investigated.

We analyzed members of non-heterocystous blue-green algae belonging to Subgroup 3, heterocystous cyanobacteria belonging to Subgroups 4 and 5 and unclassified filamentous cyanobacteria isolated from a lake in the proximity of Edmonson Point in Antarctica and from hypersaline habitat in Pantelleria.

2. Results and discussion

The yield of RPS and CPS showed large variations from one strain to another (Table 1). Generally speaking, under the growth conditions tested, RPS were more abundant than CPS, or in a quite similar amount, with the sole exceptions of *Anabaena torulosa* and *Scytonema hofmanni*, whose CPS were 6–7 times higher than RPS. The same behaviour was observed for *Nostoc linckia* and *Anabaena* sp. 7120. Gloaguen et al. (1995) reported, for the strains they examined, that generally the RPS was formed to a lesser extent than CPS.

The production of EPS by *Spirulina* was examined for the first time, in fact the species studied before, designed as *Spirulina*, belonged to the genus *Arthrospira* (Rippka & Herdman, 1992). The biomass yield for *Spirulina* (13 g wet cells l^{-1} of culture medium, Table 1) was the highest among the species examined in this paper. This value was subjected to variations with different growth conditions, with the minimum, ca half of the yield under standard growth conditions being observed when *Spirulina* was grown

Table 2
Effect of growth conditions on cell yields and EPS production by: *Spirulina* sp., *Phormidium* sp., *Anabaena torulosa* and *Anabaena* WSAF

Spirulina sp.

MEDIA	Wet cells g l^{-}	Total EPS mg l^{-1}
Alkaline	12.5	9.0
Alkaline + $NaNO_3$ 1.25 gl^{-1}	6.0	40
Alkaline + $NaNO_3$ 4.0 gl^{-1}	6.4	7.0
Alkaline−$NaNO_3$	12	55
Alkaline−NaCl	5.7	6.8
Alkaline + K_2HPO_4 1 gl^{-1}	12.8	11.7
Alkaline−K_2HPO_4	12	30
Alkaline at 30°C	9.0	5.0

Phormidium sp.

MEDIA	Wet cells g l^{-}	Total EPS mg l^{-1}
BG11	2.0	30
$BG11_0$	0.7	8.4
BG11 + $NaNO_3$ 3 gl^{-1}	1.3	9.7
BG11 + K_2HPO_4 120 mg l^{-1}	0.8	11.0
BG11−K_2HPO_4	0.6	2.9
BG11 (12 h light, 12 dark)	0.3	5.4
BG11 without aeration	0.1	3.0

Anabaena	*A. torulosa*		*A.* WSAF	
MEDIA	Wet cells g l^{-}	Total EPS mg l^{-1}	Wet cells g l^{-}	Total EPS mg l^{-1}
BG11	1.9	22	4.2	56
$BG11_0$	1.5	10	1.6	29
BG11 + $NaNO_3$ 3 gl^{-1}	5.44	10.2	12.4	22
BG11 + K_2HPO_4 120 mg l^{-1}	2.5	24	4.0	46
BG11−K_2HPO_4	1.6	13.6	2.0	4.0
BG11 (12 h light, 12 dark)	0.37	2.4	0.9	6.0
BG11 without aeration	1.0	9.3	0.54	9.0

in the absence of NaCl (Table 2); this result is not surprising since *Spirulina*, isolated from Pantelleria island, is a halophilic species. The strain of *Spirulina* biosynthesized, under standard growth conditions, 9 mg l^{-1} of RPS, and a quite similar amount of CPS.

The cyanobacterium *Oscillaroria* sp., isolated from hard sand of Pantelleria island was studied for EPS production because, although *Oscillatoria* is a rather ubiquitous genus, that thrives in a large variety of different habitats, the Pantelleria strain was isolated from a highly alkaline environment. Recently interest in microorganism culture obtained from extreme environments has grown considerably, because they represent an innovative approach to obtain new underexplored resources (Sutherland, 1990).

One of the first exopolysaccharide studied was obtained from *Anabaena cylindrica* in 1950 s by Bishop et al. (1954), although the extraction procedure adopted might have isolated slime and/or capsule material too. Among Nostocales order several species belonging both to *Anabaena* and *Nostoc* genera were previously studied (Gloaguen et al., 1995; Morvan et al., 1997).

Among the species studied in this paper classified as *Nostocaceae, Anabaena* WSAF, isolated from the gypsum dunes at White Sands New Mexico (Gambacorta et al., 1996) and *Anabaena torulosa* were found to produce the highest level of exopolysaccharides, 56 and 22 mg l^{-1} of culture medium, respectively, that represented about 14% of total cell dry weight in both cases. While, for *A. torulosa* the highest amount was as CPS ca 83% of the total EPS (Table 1), for *Anabaena* WSAF the total polysaccharide formed almost entirely corresponded to RPS. *Anabaena* 7120 and *Nostoc linckia*, under the growth conditions tested, produced a low amount of EPS (below 1% of total cell dry weight), while the EPS synthesized by *A. sphaerica* and *A. variabilis* reached only 1.5%.

Among heterocystous cyanobacteria other than *Nostocaceae*, the best producer of EPS was

Chlorogloeopsis sp. 6912, that biosynthesized about 30 mg l^{-1} of RPS that represented about 90% of total EPS formed. In *Tolypothrix tenuis* RPS was virtually the sole polysaccharide synthesized. In *Scytonema*, as reported before, the CPS were the most abundant polysaccharides formed, reaching 90% of total EPS. Among Stygonematales, for the two species examined in the present studies, *Fischerella* and *Chlorogloeopsis* 6912, the isolation and characterization of the sheaths were previously reported (Pritzer, Weckesser & Jurgens, 1989; Schrader, Drews, Golecki & Weckesser, 1982). In both cases the authors described that the sheath materials reached 20–30% of the dry weight of the cells.

The synthesis of EPS may be often related to an impairment of balanced growth both from nutritional point of view and from physical and chemical parameters (De Philippis et al., 1991; Morvan et al., 1997). Four species analysed in this paper were chosen for studying the effects of growth conditions on total EPS formed. Two non-heterocystous species and two species belonging to *Anabaena* genus.

In the case of *Phormidium* the highest EPS production was obtained under standard growth condition (BG11 medium, continuous illumination and aeration; Table 2), and corresponded to a high level of RPS while CPS represented only 2% of total EPS. Dramatic decrease of EPS yield occurred when the strain was grown with light/dark cycles, in the absence of aeration and of phosphate. The increase in phosphate and nitrogen content in the medium and the absence of combined nitrogen had similar effects on EPS yield, giving rise to a one third decrease with respect to the amount obtained under standard growth conditions. The results obtained indicated that unfavourable growth conditions induced by both nutritional and physical factors, significantly affected the EPS productivity by *Phormidium*. Several *Phormidium* species were examined for monosaccharide composition of EPS, but no data were previously reported for the EPS yield by changing growth parameters (Gloaguen et al., 1995).

The total amount of EPS biosynthesized by *Spirulina* was similar to that formed under standard growth condition, when the nitrogen content was increased up to 4 g l^{-1} and when the phosphate content was doubled (Table 2). The absence of NaCl and temperature increase caused a small decrease in EPS production. On the contrary, lowering the nitrogen content and nitrogen lack caused a strong increase in the total amount of polysaccharide, moreover, a similar effect was observed in the absence of phosphate. In conclusion, in the case of *Spirulina* the synthesis of EPS was enhanced by nitrogen-limitation and, to a lesser extent, by phosphorus-limitation. In contrast deficiency of NaCl did not induce a significant change in EPS synthesis. Similar features were reported in the case of the *Cyanothece* by De Philippis, Margheri, Pelosi and Ventura (1993).

Changes in chemical and physical parameters affected the total EPS produced by two species of *Anabaena* (A. *torulosa* and *Anabaena* sp. WSAF), and resulted in some cases in dramatic decreases. In both cases the best medium was BG11 with continuous aeration and illumination. The absence, or a higher concentration (×2), of combined nitrogen caused in both species the decrease of EPS to ca one half. The same effect was also previously reported for *A. cylindrica* 10 C (Lama et al., 1996). While the increase in phosphate abundance in the growth medium had little influence on EPS production in both species, its absence gave rise to 90% reduction in EPS yield of *Anabaena* WSAF, as was also reported for *A. cylindrica* 10 C, and to a lesser extent in *A. torulosa* (ca one half). The light/dark cycles as well as the absence of aeration caused a dramatic decrease in EPS yield.

Among neutral sugars, glucose and galactose widely occur in the studied species. Glucose was very often the most important monosaccharide found, being in the case of the CPS of *Scytonema hofmanni* the only monosaccharide detected. Other hexoses, galactose and mannose, and the pentose xylose were also plentiful in RPS of *Anabaena sphaerica, S. hoffmanni, Anabaena* WSAF and Antarctic sp. 1, respectively (Table 3).

Amino-sugar, as glucosamine, was only found in two species of *Anabaena*, while other reports (Morvan et al., 1997) showed that the polysaccharides of several other species belonging to *Phormidium* and *Oscillatoria* genera contain this amino-sugar.

Most of the species examined posses also uronic acids as galacturonic and glucoronic, whereas galacturonic acid was prominent in *Anabaena* WSAF, *Chlorogloeopsis* sp. 6912 and *Oscillatoria* sp. with respect to glucuronic residues. In both Antarctic species an unknown uronic acid was also found.

Oscillatoria sp., *A. sphaerica, S. hofmanni* and *Fischerella muscicola* were analysed for sugar composition of both RPS and CPS. *Oscillatoria* RPS and CPS exhibited the same sugar composition; hexose, as glucose, was dominant in the released as well as in the capsular fraction (Table 3). Gloaguen et al. (1995) reported the sugar composition of two species of *Oscillatoria* and in both rhamnose and mannose monosaccharides were found at higher levels, while arabinose was absent. In our strain rhamnose and mannose were absent, while arabinose was detected. Other monosaccharides such as fucose, galactose and xylose were found as well as galacturonic and glucuronic acid.

In *A. sphaerica* RPS, the most abundant monosaccharide was galactose and the only pentose was arabinose, while in CPS no pentoses were observed and the

Table 3
Sugar composition (molar ratio) of the EPS from tested Cyanobacteria grown under standard conditions (see Section 3) (–, absent; tr, trace amounts)

Cyanobacterium	Arabinose	Xylose	Fucose	Galactose	Glucose	Mannose	Rhamnose	Glucosamine
Oscillatoriales								
Spirulina sp. CPS	–	–	–	1.0	5.8	3.6	–	–
Oscillatoria sp. RPS	2.0	3.0	1.0	2.0	7.0	–	–	–
Oscillatoria sp. CPS	2.0	1.0	1.0	1.0	3.0	–	–	–
Phormidium sp. RPS	3.5	5.5	3.4	1.0	5.7	–	2.4	–
Antarctic 1 RPS	3.7	29.7	–	3.2	11.2	–	1.0	–
Antarctic 2 RPS	1.0	–	–	–	10.0	4.0	–	–
Nostocaceae								
Anabaena torulosa RPS	4.3	73.4	2.7	1.0	2.4	–	6.0	4.7
Anabaena WSAF CPS	1.2	51.0	1.0	1.6	14.5	–	10.5	5.9
Anabaena sphaerica RPS	1.0	–	–	4.0	3.0	3.0	–	–
Anabaena sphaerica CPS	–	–	–	2.0	2.0	1.0	2.0	–
Scytonemataceae								
Scytonema hofmanni RPS	–	–	–	1.0	1.0	3.0	–	–
Scytonema hofmanni CPS	–	–	tr	tr	99.0	–	–	–
Tolypothrix tenuis RPS	1.9	–	4.5	6.1	13.3	8.9	1.0	–
Stygonematales								
Fischerella muscicola RPS	–	1.0	3.0	3.0	8.0	1.0	–	–
Fischerella muscicola CPS	1.0	3.0	1.0	2.0	4.0	3.0	–	–
Chlorogloeopsis sp. 6912 RPS	1.0	–	5.0	14.5	26.4	21.6	2.9	–

different hexoses (glucose, galactose, mannose and rhamnose) occurred in similar amounts. In *S. hofmanni*, the glucose was the only monosaccharide found in CPS, while in RPS the main sugar was mannose; glucose and galactose together represented about 40% of total saccharides. No data for this species are available in literature regarding polysaccharide composition. In *F. muscicola*, the pentose arabinose was only present in CPS while fucose and glucose increased at the expenses of mannose and xylose in RPS. Previous studies on *Fischerella* reported sugar compositions for the sheath with a similar pattern to that reported in the present study (Table 3), but uronic acids and aminosugar were also identified (Bertocchi et al., 1990; Morvan et al., 1997).

It is noteworthy that *Spirulina* sp. did not contain pentoses as sugar residues but only hexoses, such as galactose, glucose and mannose; in fact, among the species examined so far, the sheaths of *Anacystis nidulans* and *Chroococcus minutus* showed the same behaviour (Morvan et al., 1997).

In the two new species isolated from Antarctic soils, the sugar composition was very different. In Antarctic 1, the hexoses found were glucose and galactose (Table 3), whereas the pentoses, as arabinose and xylose, were more abundant and accounted for ca 70% of total monosaccharides. In Antarctic sp. 2 RPS, arabinose is the only pentose detected and the hexoses were glucose and mannose.

In all species belonging to *Phormidium* genus, previously examined, mannose was an important component of both RPS and CPS (Gloaguen et al., 1995; Morvan et al., 1997), in contrast this hexose was totally absent for the strain studied here.

In *Anabaena* species examined so far (Morvan et al., 1997) rhamnose was found only by Bishop et al. (1954) in *A. cylindrica* and in trace amounts in *A. cylindrica* 10 C (Lama et al., 1996). In *Anabaena* WSAF pentoses were the most abundant sugars, and xylose represented over 50% of total sugar residues; mannose was absent while, uronic acids and aminosugar were found. In *A. torulosa* xylose was the prevalent sugar, as for *Anabaena* WSAF, but uronic acids were not present. *A. sphaerica* showed a sugar composition different with respect to the other two species examined.

For *Chlorogloeopsis* sp. 6912 only the sheath composition was previously reported and was similar to that observed in the present study (Bertocchi et al., 1990), with the exception of xylose that is absent in our strain.

^{1}H NMR studies were carried out for the RPS of *Anabaena* WSAF and for the CPS of *Chlorogloeopsis* sp. 6912; both spectra showed complex profiles. The spectrum of *Anabaena* WSAF showed, *inter alia*, five resolved signals in anomeric region at δ 4.49 ($J_{1-2}=8$ Hz); δ 4.58 ($J_{1-2}=8$ Hz); δ 5.00 ($J_{1-2}=5$ Hz); δ 5.52 ($J_{1-2}=3$ Hz) and δ 5.59 (J not detectable) and

Table 4
Chemical shifts and coupling constants of anomeric signals in ^{1}H NMR spectra of *Anabaena* WSAF and *Chlorogloeopsis* sp. 6912 (bs, broad singlet; d, doublet)

Anabaena WSAF			*Chlorogloeopsis* sp. 6912		
Residue	δ_{1H}	$^3J_{H1-H2}$	Residue	δ_{1H}	$^3J_{H1-H2}$
A	4.49	(*d*)8	A′	4.45	(*bs*)
B	4.58	(*d*)8	B′	4.61	(*bs*)
C	4.71	(*d*)8	C′	4.73	(*d*)6
D	4.76	(*d*)8	D′	4.8	(*bs*)
E	5	(*d*)5	E′	4.9	(*d*)7
F	5.52	(*d*)3	F′	4.99	(*d*)7
G	5.59	(*bs*)	G′	4.52	(*d*)2

two partially overlapping signals at δ 4.71 ($J_{1-2}=8$ Hz) and δ 4.76 ($J_{1-2}=8$ Hz); moreover, at upfield region, two signals were also observable: a doublet at δ 1.30 indicative of 6-deoxy-sugars and a singlet at δ 2.02 indicative of presence of NAc substituents. The seven anomeric signals indicated the presence of seven different sugars, regarding type or glycosidic linkage position. The seven sugars were labelled from A to G with respect to decreasing chemical shifts (Table 4). Based on chemical shifts and coupling constants it can be assumed that residues from A to D have a β *gluco-galacto* configuration, and E residue an α *gluco-galacto* configuration. The last two signals F and G showed a downfield chemical shift whose value has not been reported in literature; therefore a precise configuration cannot be ascribed to these signals.

In the same way the ^{1}H NMR spectrum of the CPS of *Chlorogloeopsis* sp. 6912 showed seven well resolved signals in the anomeric region: at δ 4.45 (J_{1-2} not resolved); δ 4.61 (J_{1-2} nr); δ 4.73 ($J_{1-2}=6$ Hz); δ 4.80 (J_{1-2} nr); δ 4.90 ($J_{1-2}=7$ Hz); δ 4.99 ($J_{1-2}=7$ Hz) and δ 5.42 ($J_{1-2}=2$ Hz). Those seven residues were labelled in Table 4 from A′ to G′ as described. Chemical shifts and coupling constant values indicated that residues A′, B′ and D′ have probably a β *manno* configuration, residues C′, E′ and F′ a β *gluco-galacto* configuration and G′ residue an α *gluco-galacto* configuration.

For *Tolypothrix tenuis* the polysaccharides of the cell wall were described before, and they were shown to be composed of mannose, glucose and xylose (Bertocchi et al., 1990) while in our study the RPS of *T. tenuis* were constituted by arabinose, as pentose, and fucose, galactose, glucose, mannose and rhamnose, and glucose was the most abundant residue.

Prokaryotic biopolymers are an important material for their potential use in biotechnology and pharmaceutical fields (Sutherland, 1990; Weiner, 1997). In particular cyanobacteria seem to be a promising source for the production of 'new' exopolysaccharides, with interesting activity such as metal removal, adhesion to solid surfaces, cytostatic, antineoplastic effects, antiviral actions (Pulz & Koehler, 1994; Sutherland, 1990).

On the basis of these observations, we have tested the inhibition of avarol toxicity on *Artemia salina* by EPS produced by some cyanobacteria analyzed in this paper (Table 5). The avarol, a sesquiterpene hydroquinone, possesses several biological activities (De Rosa, De Giulio & Strazzullo, 1991).

The highest activity was found for CPS of *Spirulina* that showed an IC_{50} of 10 ppm while the lowest activity was observed with the RPS of *Anabaena* WSAF. Perhaps, the CPS of *Spirulina* sp. and to some extent of *Oscillatoria* sp. could interfer with the toxic action of avarol on *Artemia*, e.g. via trapping into this polymeric matrix.

In this report we have identified some strains that afford large amounts of EPS, namely *Phormidium* sp., *Anabaena* WSAF, *A. torulosa* and *Chlorogloeopsis* sp., which could be good candidate for the production of such molecules either through genetic manipulation, or through the selective use of physiological conditions.

It also noteworthy that growing of photosynthetic

Table 5
Inhibition of avarol toxic activity on brine shrimp (*Artemia salina*)

	Avarol 10 ppm, polysaccharide 0 ppm	Avarol 10 ppm, polysaccharide 300 ppm	Avarol 10 ppm, polysaccharide 30 ppm	Avarol 10 ppm, polysaccharide 3 ppm	IC_{50} ppm[a]
Spirulina sp. CPS	30/0[b]	5/25	13/17	18/12	10
Oscillatoria sp. CPS	30/0	7/23	13/17	20/10	16
Fischerella muscicola CPS	30/0	11/19	17/13	18/12	37
Anabaena torulosa RPS	30/0	8/22	18/12	25/5	54
Phormidium sp. RPS	30/0	14/16	17/13	22/8	139
Chlorogloeopsis sp. 6912 CPS	30/0	17/13	22/8	28/2	198
Anabaena WSAF RPS	30/0	16/14	20/10	23/7	226

[a] 50% inhibiting concentration of avarol toxicity.
[b] Deaths/survivals *Artemia salina*.

cyanobacteria is cheaper when compared to other microorganisms (Bertocchi et al., 1990; Morvan et al., 1997). Moreover in the case of *Anabaena* WSAF, *Phormidium* and *Chlorogloeopsis* sp., whose polysaccharides were released in the medium, their recovery is more simple.

In conclusion the present paper confirmed that the studies of cyanobacterial exopolysaccharides not only provided further information on cyanobacteria metabolism but also is relevant for the identification of new products of biological interest.

Exopolysaccharides found in the species examined are formed of different monosaccharides in numbers from 3 up to 7 neutral units depending on the species; only one case of homopolysaccharide was found in *Scytonema hoffmanni* CPS, that was a glucan.

No sugar seems to characterize a particular cyanobacterial group, since the sugar composition is qualitatively and quantitatively different for different species within a given genus. For example (i) *Anabaena* WSAF, and *A. torulosa* showed a quite similar neutral sugar composition that was different in *A. sphaerica*, (ii) in *A. torulosa* the uronic acids were absent, and (iii) in *A. cylindrica* 10 C EPS the mannose, which lacked in *Anabaena* sp. WSAF and *A. torulosa* EPS was instead present. On the other hand it must be noted that the sugar composition can slightly vary in the same microorganism, especially with the age of the culture (Gloaguen et al., 1995). The present study confirms that the sugar composition of EPS seems to be of limited use as a taxonomic marker of cyanobacteria. Their chemical structures are diverse and complicated, and they exhibit activity against certain biological systems. Due to this biological activity they may be used as analytical tools for biochemistry.

3. Experimental

3.1. Organisms and growth conditions

Chlorogloeopsis fritschii, strain PCC 6912; *Tolypothrix tenuis*, strain PCC 7101, *Scytonema hofmanni*, strain PCC 7110, and *Microchaete* sp., strain PCC 7126 were obtained from the Pasteur Culture Collection (PCC), Institut Pasteur, Paris (France). *Spirulina* sp. and *Oscillatoria* sp., isolated from Pantelleria Island, were from the culture collection of our Institute (Romano, Nicolaus, Lama, Manca & Gambacorta, 1996). *Anabaena torulosa*, strain DSM B2679 was obtained from the Sammlung von Algen Kulturen (German Culture Collection). *F. muscicola* (UTEX 1829), *N. linckia, A. sphaerica* and *Phormidium* sp. were kindly provided by Prof. C.P. Wolk (Gambacorta et al., 1996). *Anabaena* WSAF sp. was kindly provided by Prof. R. Webb (Gambacorta et al., 1996). *A. variabilis*, strain ATCC 29211, was obtained from the American Type Culture Collection. *Anabaena*, strain 7120, was kindly provided by Prof. W.J. Buikema. Antarctic sp.(1, 2) isolated from samples collected from a lake near Edmonson Point in Antarctica were from the Culture Collection of our Institute (Nicolaus, Manca, Panico, Lama, Esposito & Gambacorta, 1997). The strains were cultivated photoautotrophically usually at 25°C. Antarctic spp. were grown at 11°C in BG11 medium (Rippka, 1988). *A. variabilis, A. sphaerica, Anabaena* 7120, *Microchaete* sp., *T. tenuis, C. fritscii, S. hofmanni*, and *F. muscicola*, were grown in BG11o medium (BG11 without $NaNO_3$) at pH 7.8. *N. linckia* was grown in 8-fold diluted Allen and Arnon medium (Winkenbach, 1972). *Spirulina* sp. and *Oscillatoria* sp. were grown in the alkaline medium [ALK, Rippka, 1988] at pH ca 10 (standard growth conditions). *Spirulina* sp. was grown also with different concentration of $NaNO_3$ (4, 1.25 or 0 g l^{-1}); without phosphate; with high phosphate concentration (1 g l^{-1}); without NaCl. *A. torulosa, Anabaena* WSAF, and *Phormidium* sp. were grown usually in BG11; BG11 plus $NaNO_3$ (3 g l^{-1}); BG11o; BG11 with high concentration of phosphate (120 mg l^{-1}) and without phosphate.

Cultures (1 l) were grown, unless otherwise stated, for 4 weeks in 1.5 l bottle illuminated with cool white fluorescent light (1.500 lux) and were gassed continuously by a stream of air (250 l h^{-1}); CO_2 was fluxed discontinuously in order to maintain the pH at the values indicated. Cells were harvested by filtration through a filter paper and the filtrates were used for RPS analyses. Freshly harvested cells were suspended in 100 ml 20 mM Na-Pi buffer, pH 7.0 and incubated at 60°C for 10 h. The cells were removed by centrifugation and the supernatants were used for CPS slime analyses.

3.2. Purification and characterization of EPSs (supernatant RPS and slime CPS)

RPS supernatants and CPS slimes were passed through a 100-kDa membrane filter (Amicon Millipore, Beverly, Ma, USA) to concentrate the polymer to a final volume of ca 50 ml. The polymer solns were dialysed for 2 days against tap water. Further purification of the polymer was achieved by EtOH precipitation (1 volume). The alcoholic soln was kept at −18°C overnight and then centrifuged at 15,300 *g* for 30 min. The pellet was dissolved in hot water (1/10 initial volume) and the soluble fraction accounted for 95% of the total polymers. The same procedure was repeated. The final aq. soln was dialyzed against tap water (48 h) and distilled water (20 h), lyophilized and weighed. The sample was tested for carbohydrate, protein and nucleic acid contents as described in Manca et

al. (1996). Carbohydrate content was determined according to the method of Dubois, Gilles, Hamilton, Rebers and Smith (1956) reading absorbance at 490 nm and using glucose as a standard. Pyruvate was detected after polysaccharide hydrolysis (100°C, 3 h) using a soln of 0.5% w/v of 2,4-dinitrophenylhydrazine in 2 M HCl (Duckworth & Madden, 1993). Sulfate presence was tested by the method of Silvestri, Hurst, Simpson and Settine (1982).

Hydrolysis of EPSs was performed with 2 M TFA at 120°C for 2 h. Sugar components were identified by TLC and HPAE-PAD using sugar standards for identification and calibration curves. TLC were developed with the following solvent systems: (a) Me_2CO–*n*-BuOH–H_2O (8:2:2) for neutral sugars; (b) *n*-BuOH–H_2O–AcOH (3:1:1) for acidic sugars; (c) *n*-BuOH–EtOH–H_2O (5:3:2) for oligosaccharides (Stahl, 1990). Sugars were visualized by spraying the TLC plates with α-naphthol. HPAE-PAD Dionex equipped with Carbopac PA1 column, was eluted isocratically with: (a) 15 mM NaOH for neutral sugars, and (b) 100 mM NaOH and 150 mM NaAcOH for acidic sugars (Clarke, Sarabia, Keenleyside, MacLachlan & Whitfield, 1991).

3.3. Biological assay

The inhibition of avarol toxic activity on brine shrimp (*Artemia salina*) was performed in triplicate using 10 animals, for each dose, in artificial sea water (Meyer et al., 1982). Briefly, 10 ppm of avarol dissolved in DMSO (1% of final volume) were added to each vial containing the polysaccharides in three different doses (300, 30, and 3 ppm) and for each dose survivor shrimps were counted after 24 h and data were statistically analyzed by the Finney program which yelds IC_{50} values (Finney, 1971).

Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker AMX-500 instrument at 500.13 MHz and 343 K of temperature. Before analysis, samples were exchanged twice in D_2O with intermediate lyophilization and then dissolved in 500 ml of D_2O to a final concentration of 20 mg ml^{-1}. Chemical shifts were reported in part per million relative to sodium 2,2,3,3-d_4-(trimethylsilyl)propanoate (Agrawal, 1992).

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