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ORIGINAL ARTICLE

Cyanobacteria from benthic mats of Antarctic lakes as a source of new bioactivities

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Abstract

Aims: To exploit the cyanobacterial diversity of microbial mats growing in the benthic environment of Antarctic lakes for the discovery of novel antibiotic and antitumour activities.

Methods and results: In all, 51 Antarctic cyanobacteria isolated from benthic mats were cultivated in the laboratory by optimizing temperature, irradiance and mixing. Productivity was generally very low (≤60 mg l^{-1} d^{-1}) with growth rates (μ) in the range of 0·02–0·44 d^{-1}. Growth rates were limited by photosensitivity, sensitivity to air bubbling, polysaccharide production or cell aggregation. Despite this, 126 extracts were prepared from 48 strains and screened for antimicrobial and cytotoxic activities. Seventeen cyanobacteria showed antimicrobial activity (against the Gram-positive *Staphylococcus aureus*, the filamentous fungus *Aspergillus fumigatus* or the yeast *Cryptococcus neoformans*), and 25 were cytotoxic. The bioactivities were not in accordance with the phylogenetic grouping, but rather strain-specific. One active strain was cultivated in a 10-l photobioreactor.

Conclusions: Isolation and mass cultivation of Antarctic cyanobacteria and LC-MS (liquid chromatography/mass spectrometry) fractionation of extracts from a subset of those strains (hits) that exhibited relatively potent antibacterial and/or antifungal activities, evidenced a chemical novelty worthy of further investigation.

Significance and impact of the study: Development of isolation, cultivation and screening methods for Antarctic cyanobacteria has led to the discovery of strains endowed with interesting antimicrobial and antitumour activities.

Introduction

Micro-organisms are the richest source of novel bioactive compounds. Historically, most bioactive microbial products have been obtained from two taxonomic groups (actinomycetes and filamentous fungi) and mainly from terrestrial habitats. In recent decades, the diffusion of bacterial resistance to antibiotics has stimulated the exploration of new environments and the screening of less exploited microbial groups endowed with a more versatile secondary metabolism such as myxobacteria (Gaspari *et al.* 2005) and cyanobacteria (Burja *et al.* 2001). To achieve this, the development of specific isolation and cultivation methods and the opportunity to access geographically diverse sampling areas and different ecological niches are of fundamental importance. In this context, a joint academic and industrial research project [MICROMAT EU Project BIO4-98-0040 (http://www.nerc-bas.ac.uk/public/mlsd/micromat)] was carried out aimed at the characterization and

biotechnological exploitation of the metabolic diversity of bacteria and fungi living in microbial mats at the bottom of Antarctic lakes (Tindall *et al.* 2000; Brambilla *et al.* 2001; Van Trappen *et al.* 2002; Taton *et al.* 2003, 2006a,b; Marinelli *et al.* 2004). Antarctica is the coldest and windiest continent; it is remote, hostile and uninhabited and it offers, as well as its surrounding seas, a unique opportunity to investigate and exploit the biodiversity of unexplored microbial communities (Tindall 2004). The extreme climate of Antarctica has favoured the evolution of indigenous species and novel biochemical adaptations. The Antarctic benthic mats, which have accumulated for thousands of years virtually undisturbed, due to the extreme climatic conditions and the absence of higher metazoans, are dominated by cyanobacteria, including many taxa that have never been isolated and/or cultivated before. In these dense microbial communities, the production of antibiotics and toxins may confer a competitive survival advantage (Wiegand and Pflugmacher 2005). Although the search for bioactive products (i.e. antitumour, antifungal, antibacterial and antiviral molecules) from cyanobacteria has intensified during the last few decades (Burja *et al.* 2001, 2003), to our knowledge, this is the first report on mass cultivation and pharmaceutical screening of a significant number of Antarctic cyanobacteria. The work aimed at an industrially oriented characterization of the isolated cyanobacteria by testing their bioactivities with the validated protocols of a pharmaceutical company, and by evaluating their growth capacity in order to select strains which, besides interesting bioactivities, were endowed of a good mass cultivation potential, necessary to produce high amounts of bioactive biomass.

Materials and methods

Strain isolation and characterization

Fifty-nine cyanobacterial strains were isolated from 27 benthic microbial mat samples (Fig. 1) collected during the Antarctic summers 1997–1998 and 1998–1999 from 23 lakes and ponds in the Larsemann Hills, Bølingen Islands, Vestfold Hills, Rauer Islands and the McMurdo Dry Valleys. Isolation methods and morphological-molecular characterization of these strains have been described in detail elsewhere (Taton *et al.* 2006b). From the molecular analysis, several OTUs (Operational Taxonomic Unit) were defined as groups of partial 16S rRNA gene sequences that exhibit more than 97·5% similarity, using the *Escherichia coli* positions 405–780, not taking into account indels and ambiguous bases (Stackebrandt and Goebel 1994; Taton *et al.* 2003, 2006a).

Figure 1 Mat from a lake in the Larsemann Hills showing a finely laminated structure that has accumulated over several thousand years. Microbial mats were collected using a gravity corer with a clear plastic core tube. Samples for cyanobacteria isolation, mass cultivation and screening were taken from the living surface layer of the mat.

Mass cultivation

Fifty-one out of the 59 isolates were mass cultivated in the laboratory to obtain sufficient biomass for bioactivity screenings. The remaining eight strains grew too slowly to allow mass cultivation during this project. The 51 strains were cultivated in batch under sterile conditions in 500–1100 ml glass tubes bubbled with air/CO_2 (98/2, v/v) or in 1000 ml Erlenmeyer flasks (400 ml culture volume) kept in an orbital shaker flushed with air/CO_2 (95/5, v/v). Continuous light was provided by daylight fluorescent tubes (Osram Lumilux L 58W) and its intensity ranged from 10 to 30 μmol photons m^{-2} s^{-1}, except for few strains which were cultivated at about 90 μmol photons m^{-2} s^{-1}. PAR (Photosynthetic Active Radiation) irradiance was measured using an Li185A quantum meter equipped with an Li190SB cosine quantum sensor (Li-Cor, Lincoln, NE, USA). Temperature was maintained at 20 ± 2°C for all the cultures. Freshwater cyanobacteria were cultivated in BG11 (Rippka *et al.* 1979) or BG11 without NO_3^- ($BG11_0$), added with $NaHCO_3$ (0·5%) as a

buffer. ASNIII (Waterbury and Stanier 1981) at half concentration was used for strains isolated from saline lakes. Bacterial contamination of the cultures was evaluated by observation using an optical microscope. Biomass was harvested at the end of the linear growth phase by filtration on a nylon net and carefully washed with saline solution (1 g l^{-1} for cultures in BG11 and 4 g l^{-1} for those in half strength ASNIII) under vacuum. Contamination was very low and was significantly reduced after the washing. When high amounts of polysaccharide were produced, harvesting was carried out by centrifugation. Part of the harvested biomass was dried in a stove at 100°C to constant weight in order to determine its water content and calculate the productivity (mg l^{-1} d^{-1}) on the basis of the dry weight. The productivity was calculated as the biomass concentration increase from inoculum to harvesting time (end of the linear growth phase). At 20–22°C, three strains did not grow; for 15 strains it was possible to perform only one batch. From three to six batches were carried out for 16 strains and 8–12 batches for the remaining 17 strains. The productivity was calculated as the mean of the productivities obtained in the different batches. The coefficient of variation was used to evaluate the variability in productivity.

In order to obtain a larger amount of biomass and further characterize the active molecule of one of the selected hits (ANT.L52.6), the cyanobacterium was mass cultivated in a 10-l photobioreactor constituted by a glass cylinder of 10 cm diameter and 1·5 m height, illuminated by daylight fluorescent tubes (at about 30 μmol photons m^{-2} s^{-1}). The culture was bubbled with an air/CO_2 mixture (98/2, v/v) provided through a gas diffuser placed at the bottom of the reactor. Temperature was kept at 18–20°C. The culture was carried out in semi-continuous conditions for 88 days with a harvest rate of 40–50% every 2 weeks.

Extract preparation

Five different extraction protocols were set up on the basis of biomass availability, but also considering technical drawbacks and indications obtained from the preliminary results with the extracts already processed (Table 1). Ethyl acetate extraction (48 strains) was always performed and it was preferred to methanol extraction (36 strains), as the resulting extract was less turbid and required less manipulation before the bioactivity tests. For the last group of 13 strains (see Table 1), thawing water (already obtained from 35 strains) was not produced and biomass was lyophilized.

To prepare the extracts, 1 g of biomass (dry weight) was extracted in 50 ml of solvent overnight. The suspension was then filtered on paper and the solvent evaporated under vacuum. When thawing water was obtained from frozen material, the water derived from 1 g of biomass (dry weight) was frozen again, lyophilised and extracted with 50 ml of methanol (Biondi *et al.* 2004). Finally, all the dry residues were re-dissolved in DMSO : H_2O 1 : 9 (v/v) in a proportion of 2 ml per gram of biomass extracted. When ≤0·5 g of biomass were extracted a proportion of 5 ml g^{-1} was used.

Screening for antimicrobial activities

Procedures used for the antimicrobial screening by microtiter assay in liquid have been previously described (Gaspari *et al.* 2005). The following human pathogens were used as target organisms: *Staphylococcus aureus*

Table 1 Scheme of the extraction protocols applied to the biomasses of the cultivated cyanobacterial strains

Method no.	No. strains extracted	Biomass treatment	Scheme of the extraction
1	14	Frozen, then thawed obtaining thawing water and thawed biomass	1 g biomass in ethylacetate 1 g biomass in methanol Lyophilised thawing water from 2 g of biomass in methanol
2	11	As in method 1	1 g biomass in ethylacetate Lyophilised thawing water from 1 g of biomass in methanol
3	7	As in method 1	0·5 g biomass in ethylacetate Successively, the same biomass in methanol Lyophilised thawing water from 0·5 g biomass in methanol
4	4	As in method 1	2 g biomass in methanol Successively, the same biomass in ethylacetate Lyophilised thawing water from 2 g biomass in methanol 1 g biomass in ethylacetate
5	13	Lyophilised	0·2 to 0·8 g biomass in ethylacetate Successively, the same biomass in methanol

ATCC 6538 and *Aspergillus fumigatus* ATCC 90112 from the American Type Culture Collection; *E. coli* L 47 and *Candida albicans* L 145 from the Lepetit Culture Collection c/o Vicuron Pharmaceuticals, Gerenzano, Varese, Italy; *Cryptococcus neoformans* IUM 94698 from the collection of the Istituto di Igiene, Università di Milano, Italy. *Staph. aureus* and *E. coli* were cultivated in cation-adjusted Mueller Hinton broth (CAMHB, Difco) and *C. albicans*, *A. fumigatus* and *C. neoformans* in antibiotic medium no. 3 + 2% glucose (AM3, Difco). 1×10^4 CFU or conidia ml^{-1} of each strain were inoculated into 90 μl of appropriate culture broth added to 40 μl of the cyanobacterial extract to be screened. Incubation time was 18–24 h, except for *C. neoformans* and *A. fumigatus* (48 h). Incubation was carried out under aerobic conditions at 35°C, except for *A. fumigatus* (30°C). Optical density at 620 nm was measured to detect pathogen growth inhibition. One point test was used to select the 'active' strains, i.e. those inhibiting more than 80% of the pathogen growth in comparison with the control (set as 100%) when only DMSO/H_2O was added to the pathogen inoculum. The broth micro-dilution method was used to confirm positive broths and to assay their potency (Gaspari *et al.* 2005). The activity is expressed as grams of extracted biomass (dry weight) necessary to inhibit the growth of the target organism in 1 l of inoculated medium of at least 80% compared to the control (Biondi *et al.* 2004).

Cytotoxic assay

Human cervix epitheloid carcinoma cells (HeLa cells), obtained from the Istituto Zooprofilattico di Brescia, Italy, were continuously cultured in a complete medium (RPMI 1640 supplemented with 10% foetal calf serum, Penicillin 100 units ml^{-1} and Streptomycin 100 μg ml^{-1}) at 37°C and 5% CO_2 and split at confluence using trypsin. Cells for testing were seeded in microtiter plates at a density of 10^5 cells per well. 90 μl of complete medium plus 10 μl of the cyanobacterial extract or control DMSO/H_2O solutions were added. Plates were incubated at 37°C and 5% CO_2 for 24 h. The cells were then washed with 200 μl per well of a phosphate buffer solution at pH 7·3 and pulsed for 4 h with ^{3}H-thymidine 0·1 μCi (Amersham TRK61) in 100 μl per well of complete medium without serum. Cells were trypsinized and harvested on a glass fibre filter with a semi-automated cell harvester (Wallac, Gaithersburg, MD, USA). The incorporated radioactivity was measured using a Betaplate scintillation counter (Wallac). Those samples able to inhibit 40% cell thymidine uptake relative to the control were flagged as cytotoxic. The broth micro-dilution method was used to confirm positive broths and to assay their potency (Marinelli *et al.* 2004; Gaspari *et al.* 2005).

The activity is expressed as the concentration of extracted biomass (dry weight) necessary to inhibit 40% cell thymidine uptake relative to the control.

Liquid chromatography and mass spectrometry of active fractions

Extracts were fractionated by high performance liquid chromatography (HPLC) using a Waters Chromatograph (Milford, MA, USA) on a Waters Simmetry-shield C18, 5 μm (250 × 4·6 mm) eluted at 1 ml min^{-1} flow rate at room temperature. Elution followed a linear gradient from 9% to 85% of phase B in 28 min and isocratic at 85% for the last 4 min. Phase A was acetonitrile: 20 mM ammonium formate buffer pH = 4·5, 5 : 95 v/v, and phase B was acetonitrile (HPLC grade). Detection was performed by a photodiode array from 220 to 600 nm and by mass spectrometer. The eluent from the column was split in a ratio 10 : 90 and the larger amount (about 900 μl min^{-1}) was diverted to an ultraviolet detector and then collected in a microtiter support for further assays (1 min collection for each testing tube: 32 tubes). The remaining 100 μl min^{-1} were diverted to the ESI interface of the ion trap mass spectrometer (ThermoQuest, Finningan MAT, San Jose, CA, USA). Mass spectrometric analysis was performed under the following conditions. Sample inlet: sheath gas (nitrogen) 60 psi; auxiliar gas (nitrogen) 5 psi; capillary heater 210°C. Sample inlet voltage setting: polarity both positive and negative; ion spray voltage ±5 kV; capillary voltage ±19 V. Scan conditions: maximum ion time 200 ms; ion time 5 ms; full microscan 3; scan range: 150–2000 *m/z*, both positive and negative polarity. Duration of acquisition: 29 min (from minute 3 to minute 32 of the chromatographic analysis). Bioautographies of the HPLC fractions were performed with test organisms in liquid microtiter plate assay as described above.

Results

Growth features of Antarctic cyanobacteria

The growth of the 51 isolates was tested at three different temperature ranges: 7–10, 20–22 and 28–30°C. At 7–10°C, all the strains were able to grow, but at very low rates. This temperature range was adopted for maintenance. At 20–22°C, 48 out of the 51 strains were able to grow, and sufficient biomass for bioactivity assays could be produced. Three did not grow, but remained alive. At 28–30°C, most of the strains died.

Table 2 reports the description of the 51 cyanobacterial strains for which laboratory mass cultivation was

Table 2 Taxonomic assignment, growth characteristics and bioactivity of each cultivated strain

OTU	Strain name	Morphospecies	Type of mixing	Aggregate formation	Photo-sensitivity	Productivity (mg l^{-1} d^{-1})	Productivity variability (c.v.%)	Extraction protocol no.	Antibacterial activities on:*	Antifungal activities on:*		Cytotoxicity to HeLa cells†
									Staphylococcus aureus	*Aspergillus fumigatus*	*Cryptococcus neoformans*	
16ST01[New]	ANT.LPR.2	*Pseudophormidium* sp./ *Schizothrix* sp.	AB‡	no	no	100	50	4	7·8 g l^{-1} (E§)	0	1·0 g l^{-1} (E)	0·8 g l^{-1} (E)
	ANT.LPR.3	*Pseudophormidium* sp./ *Schizothrix* sp.	AB	no	no	90	50	4	7·8 g l^{-1} (E)	0	7·8 g l^{-1} (E)	1·6 g l^{-1} (E)
	ANT.LG2.1	*Pseudophormidium* sp./ *Schizothrix* sp.	AB	no	no	90	40	4	0	0	7·8 g l^{-1} (E)	3·1 g l^{-1} (E)
	ANT.LG2.2	*Pseudophormidium* sp./ *Schizothrix* sp.	AB	no	no	130	40	4	15·6 g l^{-1} (E)	0	2·0 g l^{-1} (E)	0·4 g l^{-1} (E)
	ANT.L52B.4	*Pseudophormidium* sp./ *Schizothrix* sp.	AB	no	slight	40	40	1	0	0	0	0
16ST02	ANT.PE.3	*Pseudophormidium* sp./ *Schizothrix* sp.	OS¶	no	strong	10	60	5	0	0	0	0
	ANT.L52.1	*Leptolyngbya* cf. *fragilis*	AB	no	strong	20	110	2	0	0	0	3·1 g l^{-1} (E)
	ANT.RI8.1	*Leptolyngbya* cf. *fragilis*	AB	no	slight	30	50	2	0	0	0	0
16ST03[New]	ANT.L52.4	*Phormidium priestleyi*	AB	yes	no	40	50	1	0	1·0 g l^{-1} (M**)	0·5 g l^{-1} (M)	0
	ANT.LG2.4	*Phormidium priestleyi*	AB	no	slight	30	50	2	0	0	0	6·3 g l^{-1} (W††)
	ANT.L52.6	*Phormidium priestleyi*	AB	no	slight	40	30	1	62·5 g l^{-1} (E)	1·0 g l^{-1} (M)	1·0 g l^{-1} (M)	3·1 g l^{-1} (M)
16ST04	ANT.LPR.5	*Phormidium priestleyi*	AB	no	slight	40	80	1	0	0	0	3·1 g l^{-1} (E)
	ANT.LPR.6	*Phormidium priestleyi*	AB	no	slight	30	140	2	15·6 g l^{-1} (E)	0	0	1·6 g l^{-1} (E)
16ST05[Ant]	ANT.L66.1	*Phormidium priestleyi*	AB	no	slight	20	n.d.‡‡	5	0	0	0	0
16ST06[New]	ANT.LACV5.1	*Phormidium priestleyi*	AB	no	no	40	30	1	0	0	0	0
16ST07[New]	ANT.L53B.1	*Leptolyngbya frigida*	AB	no	slight	50	50	1	0	0	0	0
	ANT.L52.3	*Leptolyngbya frigida*	AB	no	slight	50	70	1	0	0	0	6·3 g l^{-1} (E)
	ANT.L8.1	*Leptolyngbya frigida*	AB	no	slight	50	40	1	0	0	0	6·3 g l^{-1} (E)
	ANT.L53B.2	*Leptolyngbya frigida*	AB	no	slight	30	30	2	0	0	0	12·5 g l^{-1} (W)
	ANT.L52B.3	*Leptolyngbya frigida*	AB	no	slight	50	50	1	0	0	0	0·4 g l^{-1} (M)
	ANT.REIDJ.1	*Leptolyngbya frigida*	OS	no	slight	10	n.d.	5	0	0	0	0
16ST08[New]	ANT.L52.2	*Leptolyngbya frigida*	OS	no	strong	10	n.d.	5	0	0	0	0
16ST09[New]	ANT.LMA.1	*Leptolyngbya frigida*	OS	no	slight	10	n.d.	5	0	0	0	0
	ANT.L70.1	*Leptolyngbya frigida*	OS	no	strong	10	n.d.	5	0	0	0	0
16ST10	ANT.L61.2	*Phormidium priestleyi*	AB	no	slight	50	50	1	62·5 g l^{-1} (E)	0	0	0
	ANT.LJA.1	*Leptolyngbya frigida*	AB	no	slight	20	n.d.	5	0	0	0	0
16ST11[Ant]	ANT.LG2.3	*Leptolyngbya antarctica*	AB	no	slight	10	100	3	3·1 g l^{-1} (E)	0	0	0·3 g l^{-1} (E)
	ANT.L67.1	*Leptolyngbya antarctica*	OS	no	strong	10	n.d.	5	0	0	0	0
	ANT.L18.1	*Leptolyngbya antarctica*	OS	no	slight	10	n.d.	5	0	0	0	0
	ANT.LG2.5	*Leptolyngbya antarctica*	AB	no	strong	20	120	5	62·5 g l^{-1} (M)	0	0	0
	ANT.L18.2	*Leptolyngbya antarctica*	OS	no	slight	10	n.d.	5	62·5 g l^{-1} (M)	0	0	0
	ANT.LFR.1	*Leptolyngbya antarctica*	OS	no	slight	10	n.d.	5	0	0	0	0

Table 2 *Continued*

OTU	Strain name	Morphospecies	Type of mixing	Aggregate formation	Photo-sensitivity	Productivity (mg l^{-1} d^{-1})	Productivity variability (c.v.%)	Extraction protocol no.	Antibacterial activities on:*	Antifungal activities on:*		Cytotoxicity to HeLa cells†
									Staphylococcus aureus	*Aspergillus fumigatus*	*Cryptococcus neoformans*	
	ANT.LWA.1	*Leptolyngbya antarctica*				n.g.§§						
	ANT.LWAV6.1	*Leptolyngbya antarctica*				n.g.						
16ST12	ANT.LAC.1	*Leptolyngbya antarctica*	AB	no	strong	10	60	3	0	0	0	0·2 g l^{-1} (E)
	ANT.LACV6.1	*Leptolyngbya antarctica*	AB	no	strong	20	110	2	0	0	0	3·1 g l^{-1} (E)
16ST13^{Ant}	ANT.BFI.1	*Leptolyngbya antarctica*				n.g.						
16ST14	ANT.PE.1	*Phormidium murrayi*	AB	yes	no	20	40	2	62·5 g l^{-1} (E)	0	0	3·1 g l^{-1} (E)
	ANT.LACV5.2	*Phormidium murrayi*	AB	yes	no	20	n.d.	3	0	0	0	0
	ANT.PE.2	*Phormidium murrayi*	AB	yes	no	20	n.d.	2	0	0	0	0
16ST16	ANT.L52B.1	*Nostoc* sp.	AB	no	slight	50	70	1	15·6 g l^{-1} (E)	0	15·6 g l^{-1} (E)	0·8 g l^{-1} (E)
	ANT.LPR.1	*Nostoc* sp.	AB	no	slight	50	90	1	0	0	125 g l^{-1} (W)	0·8 g l^{-1} (W)
	ANT.L61.1	*Nostoc* sp.	AB	no	strong	40	50	1	0	0	0	1·6 g l^{-1} (W)
	ANT.LG2.6	*Nostoc* sp.	AB	no	no	80	30	2	62·5 g l^{-1} (E)	0	31·3 g l^{-1} (E)	3·1 g l^{-1} (E)
	ANT.L34.1	*Nostoc* sp.	AB	no	strong	10	70	3	25·0 g l^{-1} (M)	0	0	0
	ANT.L36.1	*Nostoc* sp.	AB	no	slight	40	50	1	62·5 g l^{-1} (E)	0	62·5 g l^{-1} (E)	6·3 g l^{-1} (E)
16ST17^{New}	ANT.L52B.2	*Calothrix* sp.	OS	yes	no	30	70	2	0	0	0	0
16ST18	ANT.LPR.4	*Calothrix* sp.	OS	yes	no	20	n.d.	3	0	0	0	2·5 g l^{-1} (E)
n.d.	ANT.L52.5	*Calothrix* sp.	OS	yes	no	10	70	3	0	0	0	2·5 g l^{-1} (E)
	ANT.L52B.6	*Calothrix* sp.	OS	yes	no	10	n.d.	3	0	0	0	3·1 g l^{-1} (W)
	ANT.LG2.7	*Calothrix* sp.	OS	yes	no	10	n.d.	5	0	0	0	0

For extraction protocol description see Table 1. For strains with antimicrobial activity, cytotoxicity refers to the active extract, whereas for strains only cytotoxic, the value reported refers to the most toxic extract.

OTU, Operational Taxonomic Unit.

*Antibacterial and antifungal activities are measured as concentration of biomass extracted necessary to inhibit 80% of target organism growth compared to the control.

†Cytotoxicity is measured as concentration of biomass extracted necessary to inhibit 40% of HeLa cell thymidine uptake compared to the control.

‡Grown with air bubbling.

§Extract in ethyl acetate.

¶Grown with orbital shaking.

**Extract in methanol.

††Extract from thawing water.

‡‡Not determined.

§§Not grown.

attempted. On the basis of the morphological characterization previously reported (Taton *et al.* 2006b), *ca* 80% of the strains belong to the Oscillatoriales and *ca* 20% to the Nostocales. Within the Nostocales, six strains were assigned to the genus *Nostoc* and five to the genus *Calothrix*. Oscillatorian strains were assigned to six morphospecies, defined in agreement with Komárek and Anagnostidis (2005). Three of them (*Phormidium priestleyi*, *Leptolyngbya frigida*, *Leptolyngbya antarctica*) were considered as endemic to the Antarctica by Komárek (1999). Sequencing of 16S rRNA gene led to the assignment of the 51 strains to 17 OTUs previously described (Taton *et al.* 2006b). As shown in Table 2, oscillatorian species are grouped in 14 OTUs (from 16ST01 to 16ST14), which include six novel (at least 2·5% dissimilarity with the sequences deposited in databases) and three exclusively Antarctic (sequences already found, but only in Antarctic samples) OTUs. All *Nostoc* strains belong to the 16ST16 OTU. Within the *Calothrix* genus, two strains were assigned to different OTUs, of which one was considered as novel. We were unable to obtain the sequences of the three remaining isolates.

At 20–22°C, 13 classes of biomass productivity of 10 mg l^{-1} d^{-1} range each were recognized. Most of the strains (43 out of 48) attained a productivity lower than 60 mg l^{-1} d^{-1}. Only one strain produced more than 120 mg l^{-1} d^{-1}. The variability of productivity among the batches was in general very high, with a coefficient of variation ranging from 40% to 100% (see Table 2). There was a certain relationship between productivity and OTU assignment. All the most productive strains belonged to the same oscillatorian OTU ($16ST01^{New}$), whereas many of the less productive strains, were grouped in the oscillatorian $16ST11^{Ant}$ OTU. Two strains of the $16ST11^{Ant}$ OTU (ANT.LWA.1 and ANT.LWAV6.1) together with the other endemic *L. antarctica* ANT.BFI.1 ($16ST13^{Ant}$), even if viable, did not grow in any of the conditions tested at 20–22°C.

One common feature which limited growth of many of the less productive isolates was their inability to grow at high irradiance (photosensitivity) (Table 2). At 40 μmol photons m^{-2} s^{-1}, 10 strains (eight Oscillatoriales belonging to different OTUs and two Nostocales), showed strong depigmentation followed by death and were considered photosensitive. When cultivated at 30 μmol photons m^{-2} s^{-1}, their productivity was lower than 40 mg l^{-1} d^{-1}. Twenty-three strains (20 Oscillatoriales and three Nostocales) showed some depigmentation and low productivity at irradiances higher than 30 μmol photons m^{-2} s^{-1} and were considered slightly photosensitive. Fifteen strains (nine Oscillatoriales, one *Nostoc* and all the five *Calothrix* isolates) were not photosensitive. Among these, four strains of *Pseudophormidium* sp. belonging to the $16ST01^{New}$ OTU could be grown at 90 μmol photons m^{-2} s^{-1} and reached the highest productivities (90–130 mg l^{-1} d^{-1}).

Another factor responsible for low productivity was sensitivity to air bubbling (Table 2): 34 cyanobacteria grew well with air bubbling, whereas the remaining 14 strains could be grown only with orbital shaking. These air-bubbling sensitive strains were among the lower producers (≤10 mg l^{-1} d^{-1}, in general). Among these, four strains were assigned to *L. frigida* and belonged to $16ST07^{New}$, $16ST08^{New}$ and $16ST09^{New}$ OTUs, other four to *L. antarctica* ($16ST11^{Ant}$) and one to *Pseudophormidium* sp. (16ST02). None of the *Calothrix* strains grew when air-bubbled, but they reached productivities of 20–30 mg l^{-1} d^{-1}, when grown with orbital shaking. Other factors that negatively influenced productivity were the production of high amounts of polysaccharide, observed in one *Nostoc* (16ST16) and in one *Leptolyngbya* cf. *fragilis* (16ST02), and the formation of large aggregates, which very likely reduce light availability to the cells, as in one *P. priestleyi* ($16ST03^{New}$) and in all the strains belonging to *Phormidium murrayi* (16ST14) and *Calothrix* sp. (Table 2).

Phormidium priestleyi strain ANT.L52.6 ($16ST03^{New}$) selected, as reported below, on account of its potent antifungal activity, was cultivated for 88 days in a 10-l photobioreactor in a semi-continuous harvesting regimen and at an average concentration of 1·2 g l^{-1}. The maximum growth rate and productivity were 0·14 d^{-1}, corresponding to a generation time of 5 days, and about 95 mg l^{-1} d^{-1}. After 3 months, contamination by a green alga was observed and thus the whole culture was harvested. Over the whole cultivation period more than 43 g of biomass (dry weight) were produced.

Bioactivities of Antarctic cyanobacteria

In all, 126 samples, prepared from 48 cyanobacterial strains, grown as described above, were assayed against a panel of human pathogens and for cytotoxicity. Except in cases of very low productivity, on average, three extraction methods, two using the biomass and one using the thawing water (see Materials and Methods and Table 1), were applied to each strain to favour the recovery of active metabolites with different molecular weights and lipo/hydrophilic properties/polarities. Ethyl acetate was found to be the most appropriate solvent to extract bioactivity from these biomasses (Table 2). For all the strains, except ANT.L52.6, only one type of extract resulted active, whereas half of the cytotoxic strains showed toxicity in more than one type of extract.

Of the 48 strains tested, 17 (35%) were active. Among them seven showed only antibacterial activity, three only

antifungal activity and seven both antibacterial and antifungal activity (Table 2). No activity was detected *vs* the Gram-negative *E. coli* and the yeast *C. albicans* (data not shown), whereas the frequency of activity against the Gram-positive *Staph. aureus* was 29%. The growth of *A. fumigatus* and *C. neoformans* was inhibited by 4% and 20% of the tested strains, respectively. When tested in an *in vitro* assay, previously developed for the rapid identification of extracts active on cancer mammalian cells (Marinelli *et al.* 2004), half of the isolates were found to be cytotoxic (Table 2). The highest frequency of antimicrobial activity was detected in *Pseudophormidium* sp. (16ST01^{New}) and *P. priestleyi* (16ST03^{New}) that belong to novel OTUs, as well as in *Nostoc* sp. (16ST16). None of the strains assigned to *Leptolyngbya* cf. *fragilis* (16ST02), *L. frigida* (16ST07^{New}, 16ST08^{New}, 16ST09^{New} and 16ST10) and *Calotrhix* sp. morphospecies appeared to be microbiologically active. Among the eight *L. antarctica* isolates (16ST11^{Ant}, 16ST12), three strains showed antibacterial activity. Four strains belonging to *P. priestleyi* or *P. murrayi* were slightly active *vs Staph. aureus*, whereas two (ANT.L52.4 and ANT.L52.6) out of the three *P. priestleyi* assigned to the 16ST03^{New} OTU showed potent antifungal activity (Table 2).

The distribution of cytotoxicity did not correlate with the pattern of antimicrobial activities.

Eight out of the 17 microbiologically inactive strains of *Leptolyngbya* cf. *fragilis, L. frigida* and *Calotrhix* sp. were cytotoxic. Among *L. antarctica,* only one of the three strains endowed with antibacterial activity and two microbiologically inactive isolates produced toxins. On the other hand, most of the microbiologically active *Pseudophormidium* sp. (16ST01^{New}), *P. priestleyi* (16ST03^{New}, 16ST04) and *Nostoc* sp. (16ST16) were also cytotoxic. As the crude extracts contain a variety of different components, at this level of screening it was not possible to establish if the same or different metabolites were responsible for the cytotoxic and antibacterial/antifungal activities. Thus, preliminary analytical studies were performed with a subset of the strains (hits) that exhibited relatively potent antibacterial and/or antifungal activities.

The fractions active against *A. fumigatus* from the strains *P. priestleyi* ANT.L52.4 and ANT.L52.6 (16ST03^{New}) showed very similar chromatographic profiles and eluted with the same retention time (from 18 to 19 min), whereas the cytotoxic fraction (present only in ANT.L52.6) was clearly separated and eluted with a retention time of 26 min. Table 3 shows the antifungal and cytotoxic activities of the different fractions from strain ANT.L52.6, measured as endpoints in the microdilution method (Gaspari *et al.* 2005). In the same liquid chromatography/mass spectrometry system, *Pseudophormidium* sp. ANT.LPR.2 (16ST01^{New}), *L. antarctica* ANT.LG2.3 (16ST11^{Ant}) and *Nostoc* sp. L34.1 (16ST16) showed that the fractions active against *Staph. aureus* eluted with similar retention times, suggesting that the three strains produced chemically similar antibacterial compounds. Cytotoxicity was associated with a peak eluting later on in the chromatogram, which was present both in *Pseudophormidium* sp. ANT.LPR.2 and *L. antarctica* ANT.LG2.3, but not in *Nostoc* sp. ANT.L34.1 (data not shown). When the mass spectra obtained from the peaks corresponding to the antifungal, antibacterial and cytotoxic activities from the selected hits so far investigated were compared with those related to active microbial products stored either in the Vicuron proprietary database on microbial products (Lazzarini *et al.* 2001) or in the commercially available Dictionary of Natural Products (Anonymous 2003), no known antimicrobial metabolite was identified, suggesting a chemical novelty at the basis of the activity of these Antarctic cyanobacteria, which needs to be further investigated.

Table 3 Activity of the raw methanolic extract of the biomass from the strain *Phormidium priestleyi* ANT.L52.6 cultivated in a 10-l photobioreactor and bioautography of HPLC fractions of the extract

Fraction (retention time in min)	Antifungal activity on *Aspergillus fumigatus**	Cytotoxicity to HeLA cells†
Raw extract	1 : 256	1 : 40
1–17	<1 : 4	no
18	1 : 4	no
19	1 : 4	no
20–25	<1 : 4	no
26	<1 : 4	1 : 20
27–32	<1 : 4	no

*Antifungal activity is measured as an endpoint in microdilution the method, i.e. the highest dilution which inhibits 80% of test strain growth.

†Cytotoxicity is measured as endpoint in microdilution method, i.e. the highest dilution which inhibits 40% of HeLa cell thymidine uptake.

Discussion

Several studies have focused on cyanobacterial diversity in microbial mats of Antarctic lakes. Nevertheless, the number of Antarctic cyanobacterial strains available in culture is limited and little is known concerning their optimum cultivation conditions, physiology and metabolic diversity. Following our recent effort to obtain a wide variety of cyanobacterial strains from this biota and characterize them at the morphological and molecular level (Taton *et al.* 2006b), this study aimed to a first evaluation of their potential as a novel source of pharmaceutically valuable compounds. An essential requirement to this end was the development of cultivation and screening techniques,

which allowed the identification of strains producing interesting bioactivities (hits), and the supply of sufficient material for the biological and chemical characterization of the active metabolites. In this work, we found that most of the cyanobacteria isolated from microbial mats in a variety of Antarctic lakes (Taton *et al.* 2006b) grew better around 20°C than at lower or higher temperatures, confirming the usual psychrotolerant – not psychrophilic – nature of Antarctic cyanobacterial communities (Seaburg *et al.* 1981; Tang *et al.* 1997; Tang and Vincent 1999). Despite the mesophilic nature of these organisms, that allowed to grow them at relatively high temperatures, productivity was generally very low (≤60 mg l^{-1} d^{-1}) with growth rates (μ) in the range of 0·02–0·44 d^{-1}, values similar to those reported for other polar cyanobacteria (Roos and Vincent 1988; Tang *et al.* 1997; Tang and Vincent 1999). Photosensitivity partially explained the low productivity and low growth rate of many strains. In particular, this was the case for strains isolated from benthic lacustrine mats under a 8–12 month ice layer and thus probably adapted to low irradiances (Hodgson *et al.* 2004). On the contrary, almost all the strains that were not photosensitive were isolated from lakes with a maximum depth of less than 1 m (Taton *et al.* 2003, 2006a), and hence they were presumably adapted to high irradiances. Other factors limiting productivity depended on the mode of cyanobacterial growth in mass culture, e.g. formation of large aggregates, sensitivity to air bubbling, and production of high amounts of polysaccharide. The high variability of productivity observed among replicated cultures of the same strain evidenced a general high sensitivity of these isolates to small variations in the culture conditions, especially for the polysaccharide-producing strains. This variability can be partly explained with the short time elapsed from strain isolation to cultivation, that could have not allowed the 'selection' of the cell population better adapted to the laboratory conditions. For these reasons, a clear-cut correlation of growth features with morphological and 16S rRNA OTU grouping was not always traceable. Acclimation to light-limited culture conditions and cultivation under relatively high irradiances in photobioreactors of higher volume under careful control of temperature, irradiance, pH and medium composition are the sequence of steps needed for the exploitation of selected strains (hits) (Tredici 2004). For this, one of these hits, *P. priestleyi* ANT.L52.6, was cultivated for 88 days in a 10-l photobioreactor, allowing the production of more than 40 g of biomass dry weight, necessary for the further bioactivity characterization. The maximum growth rate achieved was similar to that obtained in the smaller reactors, but a higher maximum productivity was achieved (95 mg l^{-1} d^{-1} *vs* 48 mg l^{-1} d^{-1}). However, in a scaling-up perspective, the low irradiance required by this organism and its low growth rate that makes it a bad competitor against contaminants, might severely limit the outdoor cultivation and exploitation of this strain.

In this work, for screening purposes, extraction methods were devised to process differing amounts of biomass produced and this allowed a first insight into the metabolic diversity of a relatively large number of cyanobacterial isolates from Antarctica. Many of them were Oscillatoriales and Nostocales belonging to novel or endemic Antarctic taxa, or showed phylogenetic relationships with other polar cyanobacteria (Taton *et al.* 2006b). Burja *et al.* (2001) reported that 15 Oscillatoriales and 41 Nostocales produced 197 and 126 different bioactive metabolites, respectively. These two subsections thus appear as the most metabolically versatile within the cyanobacteria. The widely represented chemical classes were lipopeptides, depsipeptides, macrolides, macrocycles, indoles and alkaloids. In the course of our screening, the majority of the microbiologically active strains inhibited a Gram-positive pathogen, whereas no activities were found against Gram-negative and yeast representatives. This is in agreement with the few data available in the literature about antimicrobial activity frequency in cyanobacteria screenings (Kreitlow *et al.* 1999). Cytotoxicity was common among the cyanobacteria investigated in this work. As previously reported (Namikoshi and Rinehart 1996; Burja *et al.* 2001), cyanobacteria constitute a major source of toxins. Alkaloid neurotoxins and the cyclic peptide hepatotoxins are often produced by cyanobacterial water blooms worldwide. Recently some cytotoxins have been evaluated as anticancer compounds (Liang *et al.* 2005). The pattern of antimicrobial and cytotoxic activities from our cyanobacterial isolates were not in accordance with the phylogenetic relationships, but rather specific to certain strains. This confirms our previous findings that strains with identical 16S rRNA sequences isolated either from the same lake or from different lakes may produce different patterns of bioactivity, or that strains belonging to different morphospecies and OTUs may produce the same active metabolite (Taton *et al.* 2006b). For example, in this work, only two out of the three *P. priestleyi* strains belonging to the $16ST03^{New}$ OTU inhibited *A. fumigatus* growth. The two active strains were isolated from the same lake and exhibited identical partial 16S rRNA gene sequences (Taton *et al.* 2006a,b), but only one was cytotoxic. On the contrary, a similar antibacterial activity was identified in three different morphospecies, *Pseudophormidium* sp., *L. antarctica,* and *Nostoc* sp.

The chemical diversity of the active fractions, as evidenced by the mass spectra, of the isolated Antarctic strains shows the potential of this microbial group and

confirms the utility of isolating novel strains from peculiar geographical locations before screening for bioactive compounds.

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