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A high proportion of Baltic Sea benthic cyanobacterial isolates contain apoptogens able to induce rapid death of isolated rat hepatocytes

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Abstract

To assess the potential hepatotoxicity of benthic cyanobacteria, we isolated 41 strains from the Baltic Sea. The bacteria were differentially extracted with solvents of decreasing polarity. The extracts were tested for ability to induce death of primary rat hepatocytes in suspension culture. Mainly morphological criteria were used to discriminate between cell death with apoptotic features (shrinkage, chromatin hypercondensation, budding) or necrotic features (swelling, loss of plasma membrane integrity). The 24 isolates containing hepatotoxic compounds were of the genus *Anabaena*. The non-toxic isolates were mainly *Nostoc* and *Calothrix*. The toxicity was not due to the known hepatotoxic cyanobacterial protein phosphatase inhibitors microcystin or nodularin, as demonstrated by lacking competition with microcystin for PP2A binding. Apoptotic cell death was rapid, being evident from 10 to 60 min after the addition of extract. Sometimes the initial apoptosis was followed by secondary necrosis. Three cyanobacterial extracts produced apoptosis with unusual cell morphology including actin rearrangements. It will be of interest to know if they contain substance(s) acting through novel death pathways. We conclude that benthic *Anabaena* cyanobacteria represent a rich source of apoptogenic toxins, presumably directed against competitors or predators in the aquatic environment, but obviously able also to induce cell death in mammalian parenchymal liver cells.

Keywords: Benthic cyanobacteria; Hepatocytes; Leukaemia cells; Bioactive compounds and apoptosis

1. Introduction

Cyanobacteria are photosynthetic, prokaryotic organisms. A number of cyanobacteria genera produce toxins (Carmichael, 1992). The neurotoxic anatoxins are found in strains of genera *Anabaena*, *Aphanizomenon*, *Oscillatoria* and *Trichoedesmium*, and the cyclic peptide hepatotoxins microcystin and nodularin in *Microcystis*, *Anabaena*, *Nodularia*, *Oscillatoria/Planktothrix* and *Nostoc* (Sivonen and Jones, 1999). Another hepatotoxin, the protein synthesis inhibitor cylindrospermopsin is produced by *Cylindrospermopsis raciborskii* (Runnegar et al., 1995). The cyanobacterial cyclic peptides anabaenopeptin, anabaenopeptilide and microviridin are protease inhibitors, but no defined toxic activity against mammalian cells has been reported (Ishitsuka et al., 1990; Erhard et al., 1999; Burja et al., 2001). The cyclodepsipeptides apratoxin A (Luesch et al., 2001) and analogues of dolostatin, e.g. lyngbyastatin 1 (Harrigan et al., 1998) are toxic to

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mammalian cells, but the apoptogenic effect of these compounds is incompletely studied.

Cyanobacteria may produce their toxins in order to eliminate competitors or predators. Extracts from some cyanobacterial strains have been shown to paralyse competing green algae (Kearns and Hunter, 2001) and predators such as daphnids (Rohrlack et al., 2001). The nodularins and microcystins inhibit the highly conserved eukaryotic serine-threonine protein phosphatases PP1 and PP2A (MacKintosh et al., 1990; Dawson and Holmes, 1999), thus initiating apoptosis in a wide variety of cell types (Bøe et al., 1991; Fladmark et al., 2002).

There is intense research on natural compounds from microorgansims, but the marine environment has been little exploited so far, with the exception of planktonic organisms from toxic blooms. The benthic cyanobacteria seem little, if at all, investigated.

The aim of the present study was to screen benthic cyanobacteria from the Baltic Sea for apoptogenic activity. Hepatocytes were chosen as target since liver damage is a common effect of cyanobacterial poisoning. Furthermore, morphology of hepatocytes exposed to known cyanobacterial toxins is previously described and can therefore give clues to the type of apoptogen present in an extract (Fladmark et al., 1998). We found that benthic cyanobacteria, particularly of genus *Anabaena*, were a rich source of apoptogenic activity, unrelated to protein phosphatase inhibitors like nodularin and microcystin.

2. Materials and methods

2.1. Isolation and cultivation of cyanobacteria

The cyanobacterial strains used in this study (Table 1) were isolated from sediment, sand, the surface of stones, rocks, water plants, macroalgae, mussels or molluscs from the Baltic Sea at Porkkala (Gulf of Finland). Two samples originating from Seurasaari and station 39A near Helsinki were collected with plankton net. All cyanobacteria samples were suspended in a solution of 0.9% NaCl (90 ml), with 0.114% $Na_5P_3O_{10}$ and 0.002% Tween 80 (Laine et al., 1997). After vigorous agitation (100 rpm for 1 h at 20 °C), 0.1 ml of the suspension was plated on agar plates (Z8× and Z8×S) without nitrogen source (Kotai, 1972). The plates were incubated under continuous light (15 μmol m^{-2} s^{-1}) and colonies were picked and streaked on fresh plates until pure cyanobacterial cultures were obtained. The strains were identified based on the morphology according to Castenholz (2001). Mass cultivation took place in 5-l Erlenmeyer flask that contained 3 l of liquid medium and 1% of inoculum. Flasks were aerated with filter-sterilised compressed air and illuminated with day-light lamps (AIRAM, Daylight 5000 Deluxe, Finland) 15 mol m^{-2} s^{-1} at 20 °C. The cells were harvested by centrifugation or filtration after 20–60 days of culturing and freeze-dried. Total of 43 strains were mass cultivated for the analyses.

Table 1
The sample number, genus and strain code of the cyanobacteria studied

Sample number	Genus	Strain code
1	*Nostoc sp.*	XSPORK 13A
2	*Anabaena sp.*	XSPORK 7A
3	*Anabaena sp.*	XPORK 36C
4	*Anabaena sp.*	XPORK 6A
5	*Calothrix sp.*	XPORK 20A
6	*Nostoc sp.*	XPORK 24A
7	*Nostoc sp.*	XPORK 5A
8	*Anabaena sp.*	XSPORK 7B
9	*Anabaena sp.*	XPORK 2E
10*	*Anabaena sp.*	315
11	*Anabaena sp.*	XSPORK 14D
12	*Anabaena sp.*	XSPORK 27C
13	*Anabaena sp.*	XSPORK 2A
14	*Anabaena sp.*	XSPORK 27B
15	*Calothrix sp.*	XSPORK 10A
16	*Calothrix sp.*	XPORK 36 A
18	*Calothrix sp.*	XPORK 16 B
19	*Calothrix sp.*	XSPORK 3
20	*Anabaena sp.*	XSPORK 36B
21	*Calothrix sp.*	XSPORK 4A
22	*Nostoc sp.*	XPORK 14A
23	*Anabaena sp.*	XPORK 15F
24*	*Anabaena sp.*	318
25	*Anabaena sp.*	XPORK 1C
26	*Anabaena sp.*	XPORK 1D
27	*Anabaena sp.*	XPORK 2A
28	*Nostoc sp.*	XPORK 4A
29	*Anabaena sp.*	XPORK 4D
30	*Anabaena sp.*	XPORK 6B
31	*Anabaena sp.*	XPORK 6C
33	*Anabaena sp.*	XPORK 13A
34	*Cyanothece sp.*	XPORK 13B
35	*Anabaena sp.*	XPORJK15A
36	*Nostoc sp.*	XPORK 15C
37	*Anabaena sp.*	XPORK 15D
38	*Cyanothece sp.*	XPORK 15E
39	*Anabaena sp.*	XPORK 16A
41	*Nostoc sp.*	XPORK 24B
44	*Anabaena sp.*	XPORK 34A
45	*Anabaena sp.*	XPORK 35A
46	*Anabaena sp.*	XPORK 36D
47	*Calothrix sp.*	XPORK 1A
48	*Anabaena sp.*	XPORK 5C

The strains were isolated from the coast near Porkkala, Finland at the Baltic Sea. Strains 315 (10) and 318 (24), marked with asterisks, were isolated from plankton samples from Seurasaari, Helsinki, Finland and sampling site 39A at the Baltic Sea, respectively.

2.2. Chemicals

Microcystin LR was kindly provided from Prof. Dr Bernd Jastorff, University of Bremen, Germany. Nodularin was purified from cyanobacteria (*Nodularia*) following the protocol for microcystin (Runnegar et al., 1986). Microcystin YR (MC-YR) for iodination was