A COLLECTION OF POLAR CYANOBACTERIA FOR THE EXPLORATION OF DIVERSITY AND BIOTECHNOLOGICAL APPLICATIONS

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2006: BCCM (Belgian Coordinated Collections of Microorganisms) stimulation project on the elaboration of culture collections of microorganisms that were not yet available in the current consortium: a collection of polar cyanobacteria is being built.

- A way to preserve and further characterize strains isolated during projects
- But ‘New duties’ for Quality Assurance purposes
OUTLINE

1. Antarctic strains, their origin, isolation procedure, morphological diversity, genotypic diversity,
2. Arctic strains, their origin
3. Siberian strains, their origin
4. Characterisations for the collection of polar cyanobacteria
5. First dedicated screening of about 50 Antarctic strains for bioactive compounds
1. Microbial mats in Antarctic lakes

**South Victoria Land**

Lake Fryxell, Dry Valleys

**Larsemann Hills:**

Lakes Reid, Firelight, Progress, Heart

**Rauer Islands**

**Vestfold Hills**

EC project MICROMAT, LTER Dry Valleys, AAD
Examples of Antarctic strains isolation & culture

✓ Several isolation techniques, 17 culture media, 3 incubation temperatures

→ 59 strains from 23 lakes

• 34 strains at 22°C
• 23 strains at 12°C
• 2 strains at 5°C

→ Correlated to lower growth rates at low temperatures

✓ Morphological description → 12 taxa

• Oscillatoriales: 43 strains
• Nostocales: 15 strains
• Chroococcales: 1 strain

→ In agreement with the dominance of Oscillatoriales in microbial mats

✓ 16S rRNA gene sequences (56 strains)
Morphological diversity

Oscillatoriales

S40 Phormidium murrayi (Ace lake)

S12 Pseudophormidium sp (lake Gentner)

S26 Phormidium priestleyi (lake Broknes)

S32 Leptolyngbya antarctica (lake Gentner)
Morphological diversity

**Nostocales**

S16 *Calothrix sp.* (lake Broknes)

S38 *Coleodesmium sp.* (lake Broknes)

S08 *Nostoc sp.* (lake Progress)

S60 *Petalonema sp.* (lake Gentner)
GENOTYPIC DIVERSITY Antarctic strains

**OTU (Operational Taxonomic Unit):** group of 16S rRNA gene sequences with more than 97.5% of sequence similarity for positions of *E. coli*:

405-780

21 OTUs were obtained

Each OTU might correspond to more than one species, following the bacterial genospecies definition, but is surely **distinct** from other OTUs at the **specific level** (Stackebrandt & Goebel, 1994)

Underestimation of the diversity!
Examples of Antarctic strains isolation & culture

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  ➔ 59 strains from 23 lakes
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✓ Morphological description ➔ 12 taxa
  • Oscillatoriales: 43 strains
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✓ 16S rRNA gene sequences (56 strains) ➔ 21 OTUs

→ Molecular diversity > morphological diversity
Comparison with Genbank sequences

9 OTUs are novel
3 OTUs were already found in Antarctica but only there
9 OTUs were already found outside Antarctica

→ Potential endemcity 4/7

Taton et al. 2006 J. Phycology
2. Microbial mats and water in Arctic lakes

Tundra & thermokarst lakes, Bylot Island, Nunavut, Canada

→ 27 strains isolated at Laval University
3. Siberian hypersaline lakes

Benthic microbial mats in soda lakes subjected to temperatures from $-40^\circ$ to $+40^\circ$C and unstable water regimes

$\rightarrow$ 20 strains isolated at the Winogradsky Institute, RAS
4. Characterisations for the collection of polar cyanobacteria

Tests for viability, purity and authenticity:

- 16S rRNA gene
- ITS spacer between the 16S and 23S rRNA gene
- Fingerprints by variants of HIP (Highly Iterated Palindrome)

  - GCGATTCGC overrepresented in most cyanobacteria (Robinson et al. 1995)
  - Smith et al. (1999): use of variants with 2 additional bases to obtain genomic fingerprints to distinguish closely related strains
Fig. 1. Neighbor-joining tree based on 1061 16S rRNA positions, with a Jukes-Cantor correction for multiple mutations. The most similar sequence in Genbank was extracted by Sequence Match of RDPII. Indels were not taken into account. Bootstrap values higher than 70 % are indicated besides the nodes.
ITS sequences group 7

4 sequences of 2 Prydz Bay lakes and 6 sequences from Lake Fryxell

→ 11% polymorphic positions
Genomic fingerprints with HIP variants

1. Different variants: HIP-NA, HIP-NC, HIP-NT, HIP-NC
2. Fingerprints analyzed with GelComparII by cluster analysis
**Nostoc strains S15, S33, S50**

HIPNA differentiates *Nostoc* strains with identical 16S rRNA sequences.

**HIPNA**

**16S rRNA**
Strains *Phormidium murrayi* S34-S40

HIPNA differentiates *P. murrayi* strains with identical 16S rRNA and ITS sequences.
**Leptolyngbya antarctica S17-S31-S32-S36-S37-S43**

**HIPNA**

**16S rRNA**

HIPNA does not seem to differentiate well the 6 strains with identical 16S rRNA and ITS sequences

→ Need to test new HIP variants or another strategy (MLST...)**
On-going activities

Characterization of the strains with (novel) techniques having **different levels of resolution**

→ Additional data on the **diversity** and **biogeography** of polar cyanobacteria

Possibility to test **cryopreservation**:
- Antarctic strains: 83 % viable after 6 months (DMSO)
- Arctic strains more problematic
5. Screening of the Antarctic strains for bioactive compounds

Identification of Novel Compounds
Integrated Approach

- Relevant Medical Need
- Target & Assay
- Microbial Extract Bank
- Strain Library

- HTS
- HIT

- Initial Profiling and Novelty Determination

- Lead Optimization
- In vitro & In vivo Profiling

- Developmental Candidate
- Process Development
  Scale-up & Supply
1 liter culture of 51 antarctic cyanobacterial strains and extract preparation

(Mario Tredici & Natascia Biondi, Università di Firenze)

Microbial activity profile of 126 extracts from 48 strains & selection of two hits for further characterization

(Vicuron Pharmaceuticals (Biosearch Italia))

Mass cultivation conditions

• Slow-growing (biomass productivity <60 mg L\(^{-1}\) d\(^{-1}\))
• Optimal growth at 20-22°C

Inability to adapt to high light intensity and to air bubbling cultivation limited their biomass productivity more than temperature, as they were almost all psychrotolerant, rather than psychrophilic

For 48 isolates, enough biomass produced for screenings.

126 chemically diverse extracts (ethyl acetate or methanol from biomass or thawing water) screened by a combination of HT antimicrobial assays and cytotoxicity tests.
Antimicrobial assays:

**Microtiter assay** in liquid, with measure of optical density at 620 nm to detect pathogen growth inhibition

**Active strains**: more than 80% of pathogen growth inhibition in comparison to controls (100%)

- **10⁴ CFU/ml pathogen**
- **90 µl culture broth**
- **40 µl extract to screen**

O.D. reading after 18-24 hours for *S. aureus*, *E. coli* and *C. albicans*, and after 48 hours for *A. fumigatus*, *C. neoformans*

+ **Confirmation by broth micro-dilution assays** (Gaspari et al. 2005)
Cytotoxicity to HeLa cells:

Incorporation of $^3$H-thymidine in cell cultures

Cytotoxic strains: more than 40% of inhibition relative to controls (100%) after 24H incubation
Cyanobacterial screenings

17 cyanos produced antimicrobial activities (35%)
25 were cytotoxic (50%)

29 % versus *S. aureus*
20% versus *C. neoformans*
2% versus *A. fumigatus*

No activities against *E. coli* and *C. albicans*

Bioactivities were not in coincidence with the phylogenetic relationship, but rather specific to certain strains i.e.

**Identical strains** (rRNA) isolated from the same lakes or from different lakes may produce **different patterns of bioactivity**
### Hits selected, tested in endpoint and fractionated by LC-MS

<table>
<thead>
<tr>
<th>16rRNA OTU</th>
<th>Morphotype</th>
<th>Identification</th>
<th>Hits</th>
<th>Activity on <em>S. aureus</em></th>
<th>Activity on <em>A. fumigatus</em></th>
<th>Activity on <em>C. neoformans</em></th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>16ST03 New</td>
<td>OS-II</td>
<td><em>Phormidium pristleyi</em></td>
<td>ANT. LH52.4</td>
<td>0</td>
<td>512</td>
<td>1024</td>
<td>0</td>
</tr>
<tr>
<td>16ST03 New</td>
<td>OS-II</td>
<td><em>Phormidium pristleyi</em></td>
<td>ANT. LH52.6</td>
<td>8</td>
<td>512</td>
<td>512</td>
<td>160</td>
</tr>
<tr>
<td>16ST01 New</td>
<td>OS-I</td>
<td><em>Pseudophormidium sp.</em></td>
<td>ANT. PROGRESS 2.2</td>
<td>64</td>
<td>0</td>
<td>512</td>
<td>640</td>
</tr>
<tr>
<td>16ST13 Ant</td>
<td>OS-V</td>
<td><em>Leptolyngbya antarctica</em></td>
<td>ANT. GENTNER 2.3</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>640</td>
</tr>
<tr>
<td>16ST19</td>
<td>NO-I</td>
<td><em>Nostoc sp.</em></td>
<td>ANT. LH34.1</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Microbiological activity**: the highest dilution which inhibits 80% of the pathogen growth

**Cytotoxicity**: the highest dilution which inhibits 40% of HeLa cell thymidine uptake
HPLC fractionation + LC-MS of active fractions of the two hits (antifungal activity) showed very similar chromatographic profiles (→ duplicates?), whereas the fraction active against A. fumigatus was separated from the fraction exhibiting cytotoxicity.

For the 3 other interesting strains, LC-MS showed that the fraction active against S. aureus eluted at similar retention times, suggesting that the three strains produced similar novel antibacterial compounds (Luc Jacquet, personal communication).
Cyanobacteria = Promising microbial group but somewhat difficult to cultivate

New secondary metabolites from cyanobacteria

VICURON database, Lazzarini et al. (2000) *Antonie van Leeuwenhoek* 78:399-405
Examples of bioactivities in the 5 cyanobacterial orders

Table 1. (continued)

<table>
<thead>
<tr>
<th>Order</th>
<th>Compounds</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chroococcales (11)</td>
<td>36</td>
<td>Enzyme inhibitor, cytotoxic, cell-differentiation, tumor promoter, endotoxic, hepatotoxic (6)</td>
</tr>
<tr>
<td>Pleurocapsales (1)</td>
<td>2</td>
<td>Antifungal, no activity (2)</td>
</tr>
<tr>
<td>Oscillatoriales (15)</td>
<td>197</td>
<td>Antialgal, anticancer, anti-HIV, antifeedant, antifungal, anti-inflammatory, antimicrobial, antimitotic, antiproliferative, antiviral, brine shrimp toxicity, cytotoxic, cytoskeleton disruption, herbicidal, hepatotoxic, ichthyotoxic, immunosuppressive, molluscidal, neurotoxic, no activity, PBDu binding, tumor promoter, protein kinase activator, skin irritant, sunscreen pigment, toxin (26)</td>
</tr>
<tr>
<td>Nostocales (41)</td>
<td>126</td>
<td>Anticancer, antifungal, antimalarial, anti-HIV, cardioactive, hepatotoxic, antimicrobial, antimitotic, anti-inflammatory, antiviral, cytotoxic, enzyme inhibitor, toxin, neurotoxin, pigment, no activity (16)</td>
</tr>
<tr>
<td>Stigonematales (6)</td>
<td>16</td>
<td>Antifungal, antibiotic, anticancer, antimitotic, cytotoxic, herbicidal, no activity (7)</td>
</tr>
</tbody>
</table>

Biological activities of 424 compounds from marine cyanobacteria (2001)
→ Renewed interest for biotechnological exploitation of cyanobacteria, with a steadily increasing number of cyanobacteria found to produce a variety of novel and biologically active compounds

→ Current discussion at ATCM level concerning the exploitation of Antarctic genetic resources and how this fits with the Antarctic Treaty and the Madrid Protocol
Thanks for your attention!

New Belgian Antarctic Station in Dronning Maud Land!