Overview of Last Lecture

1. Gene expression regulation
   1) Two major modes
      a) Inhibition by allosteric effector and enzyme modification
      b) Protein processing.
   2) Antisense regulation
   3) Regulation of transcription
      a) Common structures of DNA binding proteins (bacteria and eukaryotes)
      b) DNA structure & protein binding sites
      c) Negative control – repression & induction
      d) Positive control – enhancement & induction
Visiting Biotechnology Facility

When: January 23 at 1:30-2:45 PM (regular class time)

Where: Gilmore Hall Room 411
        3050 Maile Way

Who: All class attendants are required to come
Overview of Last Lecture

e) Control by two-component system
f) Regulation in eukaryotes (transcription factors & their binding elements)

2. DNA manipulation enzyme (restriction endonuclease & AND ligase)

3. Clone vector (plasmid & cosmid)
Cultivation of “Uncultured” Microbes

Why do we have to culture microorganism?

1. So, if a bacterium cannot be cultured, it cannot be identified by traditional (biochemical) methods.


3. Cultures are essential for most biotechnological purposes.

4. Most uncultured bacteria seem to differ substantially (based on 16S rRNA) from cultured isolates.
Difference of Cultured and Uncultured Marine Bacterioplankton Species

Cultured organisms: note dominance by gamma-proteobact

Uncultured organisms: many more alpha-proteobact!

Hagstrom et al. 2002.
New Culture Methods


Seawater

- 0.2 µm pore size filter

Restoration of bicarbonate buffer (introduce CO₂)

- Check sterility using direct cell counting

Direct count of the inoculum by fluorescence microscopy

- Dilute inoculum into prepared medium at 1-5 cells per ml and fill 48-well microtiter plate with 1 ml per well

- Incubate under the desired time and conditions

- Array 200µl aliquots onto a 48 sector filter manifold, stain and transfer to a microscope slide

- Screen for positive growth by fluorescence microscopy

- Identify cultures by PCR, RFLP and sequencing

- Transfer to fresh medium

- Store cultures with DMSO and/or glycerol in liquid N₂
## HTC cultivability compared to traditional cultivability counts

<table>
<thead>
<tr>
<th>Date (mo-day-yr) and location of inoculation sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inoculum sample (cells/ml)</th>
<th>Avg no. of cells/well</th>
<th>Total no. of wells inoculated</th>
<th>No. of positive wells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Culture designations</th>
<th>% Culturability&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% Culturability on nutrient-rich agar&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-21-98, J</td>
<td>$1.1 \times 10^6$</td>
<td>1.1</td>
<td>144</td>
<td>7</td>
<td>HTCC1–7</td>
<td>4.5 (1.8, 9.3)</td>
<td>―</td>
</tr>
<tr>
<td>6-5-98, J</td>
<td>$1.5 \times 10^6$</td>
<td>1.5</td>
<td>192</td>
<td>37</td>
<td>HTCC8–44</td>
<td>14.3 (10.0, 19.7)</td>
<td>―</td>
</tr>
<tr>
<td>7-6-98, 8 km</td>
<td>$3.7 \times 10^6$</td>
<td>3.7</td>
<td>192</td>
<td>62</td>
<td>HTCC45–106</td>
<td>10.5 (8.0, 13.5)</td>
<td>―</td>
</tr>
<tr>
<td>7-6-98, 25 km</td>
<td>$1.5 \times 10^6$</td>
<td>1.5</td>
<td>192</td>
<td>37</td>
<td>HTCC107–143</td>
<td>14.3 (10.0, 19.7)</td>
<td>―</td>
</tr>
<tr>
<td>6-17-99, J</td>
<td>$5.6 \times 10^6$</td>
<td>3.0</td>
<td>192</td>
<td>21</td>
<td>HTCC144–164</td>
<td>3.9 (2.4, 5.9)</td>
<td>―</td>
</tr>
<tr>
<td>10-29-99, J</td>
<td>$1.9 \times 10^6$</td>
<td>3.0</td>
<td>192</td>
<td>10</td>
<td>HTCC165–174</td>
<td>1.8 (0.9, 3.3)</td>
<td>―</td>
</tr>
<tr>
<td>12-21-99, J</td>
<td>$8.1 \times 10^5$</td>
<td>5.0</td>
<td>384</td>
<td>10</td>
<td>HTCC175–184</td>
<td>0.5 (0.3, 1.0)</td>
<td>―</td>
</tr>
<tr>
<td>1-26-00, J</td>
<td>$1.1 \times 10^6$</td>
<td>5.0</td>
<td>192</td>
<td>11</td>
<td>HTCC185–191, 193–196</td>
<td>1.2 (0.6, 2.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>4-5-00, J</td>
<td>$9.0 \times 10^5$</td>
<td>5.0</td>
<td>192</td>
<td>20</td>
<td>HTCC197–216</td>
<td>2.2 (1.3, 3.4)</td>
<td>0.15</td>
</tr>
<tr>
<td>7-12-00, J</td>
<td>$1.9 \times 10^6$</td>
<td>3.0</td>
<td>228</td>
<td>33</td>
<td>HTCC217–233, 236–251</td>
<td>5.2 (3.6, 7.3)</td>
<td>0.98</td>
</tr>
<tr>
<td>10-9-00, 8 km</td>
<td>$1.3 \times 10^6$</td>
<td>3.0</td>
<td>384</td>
<td>5</td>
<td>HTCC252–256</td>
<td>0.4 (0.1, 1.0)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

<sup>a</sup> Date and location of inoculation sample.

<sup>b</sup> No. of positive wells.

<sup>c</sup> % Culturability.

<sup>d</sup> % Culturability on nutrient-rich agar.
### 16S rRNA sequence similarities of HTCC isolates from previously uncultivated clades to the nearest neighbors in GenBank

<table>
<thead>
<tr>
<th>Clade</th>
<th>HTCC isolate</th>
<th>E. coli position^a</th>
<th>Clone</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAR11</td>
<td>150</td>
<td>524–1362</td>
<td>ZD0409</td>
<td>99.8</td>
</tr>
<tr>
<td>OM43</td>
<td>144</td>
<td>712–1386</td>
<td>OM43</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>165</td>
<td>710–1386</td>
<td>OM43</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>711–1371</td>
<td>POCPN-5^b</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>713–1362</td>
<td>OM43</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>185</td>
<td>719–1378</td>
<td>OM43</td>
<td>100</td>
</tr>
<tr>
<td>SAR92</td>
<td>148</td>
<td>716–1384</td>
<td>MB11B11</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>151</td>
<td>716–1383</td>
<td>MB11B11</td>
<td>96.9</td>
</tr>
<tr>
<td></td>
<td>153</td>
<td>713–1360</td>
<td>MB11B11</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>154</td>
<td>716–1360</td>
<td>Artic97A-6</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td>157</td>
<td>716–1351</td>
<td>Artic97A-6</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>221</td>
<td>716–1346</td>
<td>MB11B11</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>228</td>
<td>707–1266</td>
<td>SAR92</td>
<td>96.5</td>
</tr>
<tr>
<td></td>
<td>230</td>
<td>28–1537</td>
<td>MB11B11</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>234</td>
<td>28–1537</td>
<td>MB11B11</td>
<td>97.0</td>
</tr>
<tr>
<td>OM60/OM241</td>
<td>160</td>
<td>713–1383</td>
<td>OM60</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>227</td>
<td>705–1373</td>
<td>MERTZ-2CM-38</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>716–1360</td>
<td>MERTZ-2CM-38</td>
<td>96.9</td>
</tr>
</tbody>
</table>
Encapsulation of cells in gel microdroplets (PNAS, 26:15681-6)

1. Filter-sterilized Sea Water (SSW).

2. SSW amended with NaNO₃ K₂HPO₄, NH₄Cl, trace metals, and vitamins.

3. SSW amended with amino acids.

4. Marine medium (R2A, Difco).
Diluted cell suspension (0.1 ml) were mixed with 0.5 ml of preheated agarose (at 40 °C). Cell agarose mixtures were added into 15 ml of CellMix emulsion matrix and emulsified at RT for 1 min.

Flowchart of the experimental procedure. Cells captured from environmental samples were encapsulated into gel microdroplets (GMDs) and incubated in growth columns (phase I). GMDs contained microcolonies were detected and separated by flow cytometry into 96-well microtiter plates containing a rich organic medium (phase II).
Phylogenetic tree based on 16S rRNA sequences that were retrieved from GMDs from the seawater. Shown are groups of the alpha, beta, gamma, and delta subclasses of *Proteobacteria*, as well as of the *Cytophaga-Flavobacterium-Bacteroides* and relatives (CFB and *Planctomycetes* and relatives (Plan)).
Simulation of natural environment (growth chamber)

Microorganisms from sediment (marine sediment) → Mixed with warm agar (seawater) → Diffusion chamber (Membranes-exchange and restriction) → Incubate the chamber in an aquarium

Diffusion growth chamber for in situ cultivation of environmental microorganisms. A. the chamber is formed by a washer sandwiched between two 0.03 µm pore-size polycarbonate membranes. B. Growth chambers incubated on the surface of marine sediment.

Science 296:1127
Growth recovery (± SD) of microorganisms from environmental samples in diffusion chambers.

Representative colonies of marine-sediment microorganisms (compound microscope view) grown in diffusion chambers.
Significance of Cultivation

Box 1 Recognized bacterial phyla.

Represented by pure cultures
1. Acidobacteria
2. Actinobacteria
3. Aquificae
4. Bacteroidetes
5. Chlamydiae
6. Chlorobi
7. Chloroflexi
8. Chrysilogenetes
9. Coprothermobacteria
10. Cyanobacteria
11. Deferribactera
12. Dehalococoides
13. Deinococcus-Thermus
14. Dictyoglomus
15. Fibrobacteres
16. Firmicutes
17. Fusobacteria
18. Nitrospira
19. Planctomycetes
20. Proteobacteria
21. Spirochaetes
22. Synergistes
23. Thermodesulfovibrio
24. Thermomicrobiia
25. Thermotogae
26. Verrucomicrobia

Cultivated, but not-yet-pure
27. Candidate OP10
28. Candidate TM7

Not-yet-cultivated
29. Candidate marine group A
30. Candidate OP3
31. Candidate OP5
32. Candidate OP8
33. Candidate OP9
34. Candidate OP11
35. Candidate OS-K
36. Candidate termite group 1
37. Candidate TM6
38. Candidate WS1
39. Candidate WS6

A. 1987 – phylogenetic tree contains 12 phyla
B. 2000 – phylogenetic tree contains 39 phyla
C. Of the 27 new phyla, at least 16 are identified through cultivation
D. Only 11 of the currently recognized 37 bacterial phyla remain elusive to our cultivation attempts.
General Concerns for Cultivation

1. Have sufficient knowledge or imagination of the chemistry of their native, intracellular milieu. Being able to recreate viable “man-made natural” conditions for them.

2. Being patient (obvious turbidity or colonies take long time to develop).
Several molecular approaches are available for analysis of microbial communities.
rRNA secondary structure & bacterial universal primers

Van de Peer et al. 1996

Increased variability
Bacterial Primers and Designation

Baker et al., 2003
Summary of Bacterial tRNA Operon

1. 16S rRNA gene: conserved area (taxa), variable 16S regions (genera and species)
2. Spacer regions: a large degree of sequence and length variation at the levels of genus and species (the number and type of tRNA sequences)
3. The length and nucleotide sequences can be used to delineate bacterial strains and species
4. The spacer length ranges in size from ~400 – 1,400 bp with majority of length (~90%) being 400 – 800 bp.
Amplified rDNA restriction analysis (ARDRA)

1. Procedures of ARDRA

Genomic DNA → 16S rDNA primers → PCR products → 16S rDNA fragments → Loading samples → Agarose gel separation → Ethidium bromide → Pattern analysis

2. Principle of ARDRA

Restriction enzyme → U-F1 → U-R1
ADRA Analysis of 16S rRNA Genes for Isolates from Marine Sponges

Restriction Map

A: HaeIII  B: HaeIII/Sau 3AI

Phylip program – fragment analysis (UPGMA)
Identification of cultured mycobacteria in a diagnostic laboratory


↓

DNA extraction

↓

MBUZ1/MBUZ2 (~1,500 bp) for Actinomycetales

↓

Restriction Digestion

↓

Gel separation

↓

Comparison of ARDRA profiles
Identification of cultured mycobacteria in a diagnostic laboratory (cont.)

HindI (CfoI) restriction patterns of amplified mycobacterial 16S rRNA genes. Legend: M: marker (100 base pair ladder, Fermentas, Vilnius, Lithuania)

<table>
<thead>
<tr>
<th>Pattern</th>
<th>CfoI restriction fragment lengths (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>408, 385, 312, 181, 168</td>
</tr>
<tr>
<td>1'</td>
<td>408, 388, 312, 187, 160</td>
</tr>
<tr>
<td>1''</td>
<td>408, 388, 312, 196, 157</td>
</tr>
<tr>
<td>2</td>
<td>408, 387, 347, 312</td>
</tr>
<tr>
<td>3</td>
<td>408, 385, 346, 300</td>
</tr>
<tr>
<td>4</td>
<td>408, 387, 225, 190, 155, 75</td>
</tr>
<tr>
<td>5</td>
<td>408, 387, 225, 187, 160, 75</td>
</tr>
<tr>
<td>7</td>
<td>408, 362, 225, 192, 152, 75</td>
</tr>
<tr>
<td>8</td>
<td>864, 408, 368</td>
</tr>
<tr>
<td>10</td>
<td>408, 389, 347, 225, 75</td>
</tr>
<tr>
<td>13</td>
<td>365, 346, 302, 300, 106</td>
</tr>
<tr>
<td>14</td>
<td>408, 360, 346, 312, 25</td>
</tr>
<tr>
<td>15</td>
<td>422, 408, 363, 225, 25</td>
</tr>
<tr>
<td>16</td>
<td>408, 384, 344, 225, 74, 25</td>
</tr>
</tbody>
</table>

HindI (CfoI) restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.
Identification of cultured mycobacteria in a diagnostic laboratory (cont.)

Library of ARDRA profiles (combination of restriction patterns) obtained for mycobacterial species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genbank Number</th>
<th>HhaI</th>
<th>MboI</th>
<th>RsaI</th>
<th>BstUI</th>
<th>Reference strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis complex</td>
<td>X52917</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>ITG 8021, IPB 92/080</td>
</tr>
<tr>
<td>M. conspicuum</td>
<td>X88922</td>
<td>1'</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>M. intracellularare</td>
<td>X52927</td>
<td>1'</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>ITG 5913, ITG 5917</td>
</tr>
<tr>
<td>M. gastri</td>
<td>X52919</td>
<td>1'</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>M. kansasii</td>
<td>M29575</td>
<td>1'</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>ITG 8201</td>
</tr>
<tr>
<td>M. bohemicum</td>
<td>AJ277283</td>
<td>1'</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>M. haemophilum</td>
<td>U06638</td>
<td>1'</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>ITG 3065</td>
</tr>
<tr>
<td>M. malmoense</td>
<td>AF152560</td>
<td>1'</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>ITG 940611</td>
</tr>
<tr>
<td>M. szulgai</td>
<td>X52926</td>
<td>1'</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>ITG 4981</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>X52924</td>
<td>1'</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>ITG 4988</td>
</tr>
<tr>
<td>M. xenopi</td>
<td>X52929</td>
<td>1'</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>ITG 4986</td>
</tr>
<tr>
<td>M. heckeshornense</td>
<td>AF174290</td>
<td>1'</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>M. marinum</td>
<td>AF251565</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>ITG 1728</td>
</tr>
<tr>
<td>M. asiaticum</td>
<td>M29556</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>ITG 8182</td>
</tr>
<tr>
<td>M. terrae</td>
<td>X52925</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>ITG 4922</td>
</tr>
<tr>
<td>M. ulcerans</td>
<td>Z13990</td>
<td>2</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>ITG 724, ITG 1837</td>
</tr>
<tr>
<td>M. avium</td>
<td>AF306455</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>ITG 4991, ITG 2666</td>
</tr>
<tr>
<td>M. batniense</td>
<td>AJ012756</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>M. terrae-like MCRO6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>X93032</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>M. nonchromogenicum</td>
<td>X52928</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>ITG 4980</td>
</tr>
<tr>
<td>M. phlei</td>
<td>M29566</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>2'</td>
<td></td>
</tr>
<tr>
<td>M. elephantis</td>
<td>AJ010747</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>3'</td>
<td></td>
</tr>
</tbody>
</table>
Identification of cultured mycobacteria in a diagnostic laboratory (cont.)

Neighbour-joining similarity tree for 16S rRNA gene sequences of most mycobacterial species. Legend: *N. asteroides* ATCC 49872 (Genbank Z82229) was used as the outgroup. Table 1 lists the GenBank accession numbers of the sequences used to construct this tree. ARDRA patterns for *Hhal*, *Mbol*, *Rsal* and *BstUI* are listed after the species name. a: GenBank AF028712. Erroneously listed in GenBank as *M. peregrinum* (see also legend of Table 1). b: *M. gastri* clusters below 100% with *M. kansasii*, although it is generally agreed that the 16S rRNA gene sequences for *M. kansasii* and *M. gastri* are identical. This can be explained by the fact that the only available GenBank *M. gastri* sequence (X52919) contained several ambiguities. c: *M. lentiflavum*, initially not included in the manuscript is not presented in this tree. It clusters close to the branch including *M. heidelbergense*, *M. simiae*, *M. triplex* and *M. genavense*. 

Mycobacterium chelonae group II, 13-3-5-6-6
Mycobacterium branderi, 14-1-1-9
Mycobacterium celatum, 14-1-1-10
Mycobacterium intermedium, 5-4-6-1'
Mycobacterium kubae, 10-1-6-1'
Mycobacterium interjectum, 5-4-6-2'
Mycobacterium heidelbergense, 5-4-8-2'
Mycobacterium simiae, 5-7-6-2'
Mycobacterium genavense, 10-7-6-2'
Mycobacterium triplex, 10-7-6-2'
Mycobacterium concinnum, 1'-1-2-2
Mycobacterium gordoni, 8-4-2-5
Mycobacterium asiaticum, 2-1-2-5
Mycobacterium haemophilum, 1'-4-1-3
Mycobacterium tuberculosis, 1-1-1-1
Mycobacterium ulcerans, 2-1-9-1
Mycobacterium marmum, 2-1-1-1
Mycobacterium scrofulaceum, 1'-4-2-3
Mycobacterium kansasii, 1'-4-1-1
Mycobacterium gastri, *a* 1'-4-1-1
Mycobacterium bohemicum, 1'-4-1-3
Mycobacterium szulgai, 1'-4-2-1
Mycobacterium malmoeense, 1'-4-1-3
Mycobacterium intracellulare, 1'-2-2-3
Mycobacterium avium, 2-2-2-3

*Nocardia asteroides*
Identification of cultured mycobacteria in a diagnostic laboratory (cont.)

All but three of 151 mycobacterial isolates were identified with ARDRA within on average 36 hours.

Conclusions:
1. A practical short cut to full sequence determination.
2. The discriminatory power of ARDRA for identification of mycobacteria is almost as high as that of sequencing.

Drawback:
Culture-dependent
Overestimate the number of community number.

AEM, 2004, 70:202-203
Summary

• Significance of cultivation
  – New cultivation methods
  – General concerns
  – Contribution of cultivation

• Bacterial 16S rRNA gene and universal primer

• Overview of molecular approaches for microbial diversity

• ARDRA and its applications
  – Principle and procedure
  – Advantage and drawback