Molecular Approaches for Exploring Uncultured Bacterial Diversity in Extreme Environment

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Why Do We Need to Identify Microorganisms

- Only a few thousands of the estimated 100,000 of microbial species that inhabit our planet can be cultured in the laboratory.
- By identifying a microorganism or group of microorganisms we can label them as pathogenic, beneficial, industrially useful etc.
- The treatment is exceptionally difficult when the pathogen is unknown.
- Identification makes it convenient for microbiologist to know them, work with them and communicate efficiently
- It helps organize the huge amount of knowledge about them.
- Many beneficial microorganisms play a major roles in soil fertility, biogeochemical cycles, biodegradation and bioremediation of toxic wastes.
- Many large scale industrial processes like fermentation ,antibiotics, beverage production are all dependent on microorganisms.
- Microbes are present even in extreme environments like low pH, deep sea vents, polar icecaps and in hot water springs. So it is very important to identify and understand them and in what way they affect us.

Conventional methods

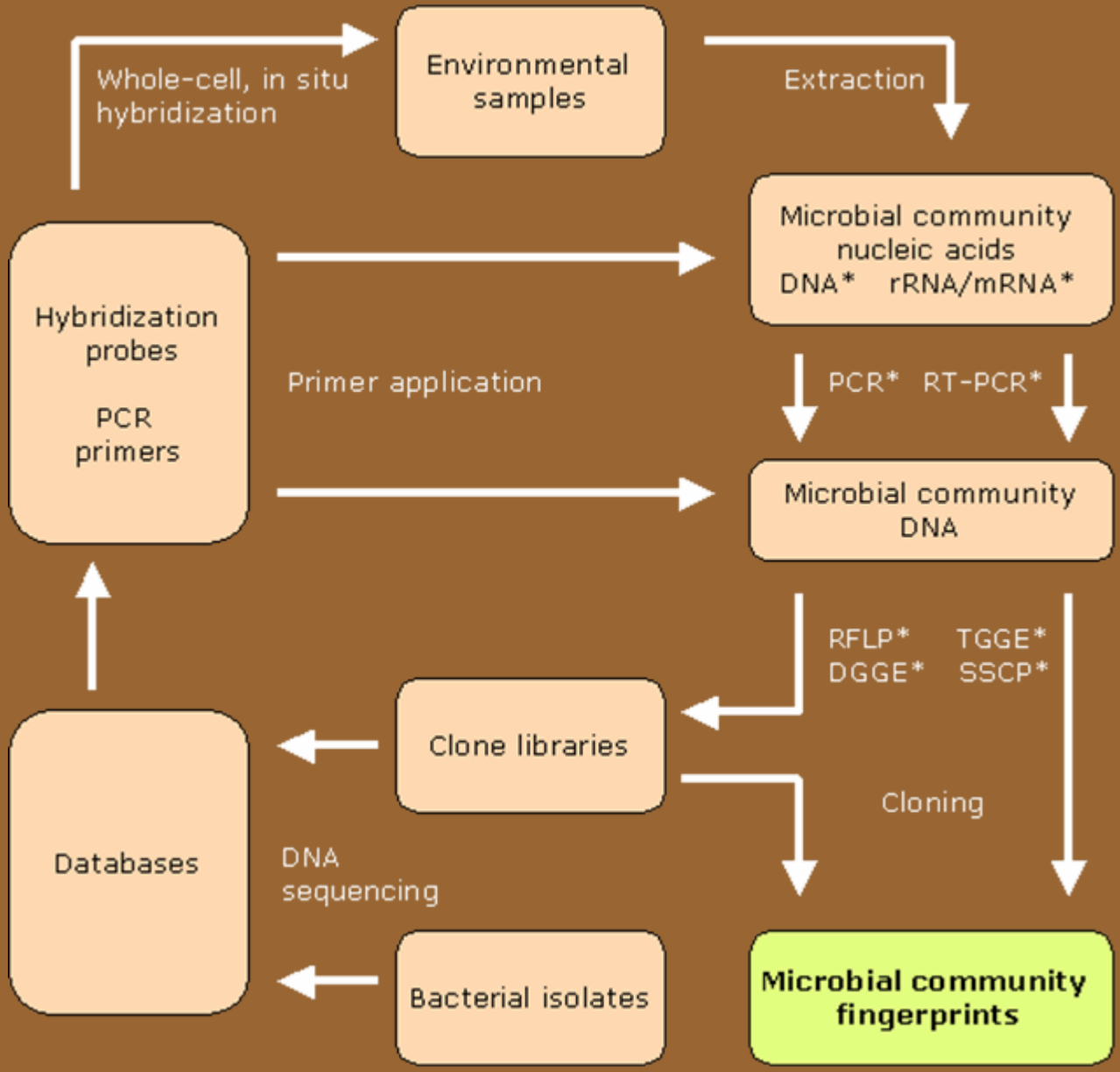
- Depend on ability to culture
- Slow, esp. for fastidious species
- Not always definitive
- Inconclusive if culture is impure

Difficulties experienced with methanogens/iron-, sulfur-oxidizers

- Slow growers
- Difficult to cultivate on solid media.
- Difficult to get isolated colonies on solid media.
- Genetic improvement of strain, taxonomic studies and phylogenetic studies have lagged behind.

Molecular identification

- Reduce reliance on culture
- Faster
- More sensitive
- More definitive
- More discriminating
- Techniques adaptable to all bacteria


Whole-cell, in situ hybridization
Environmental samples
Extraction
Microbial community nucleic acids
DNA* rRNA/mRNA*
Hybridization probes
PCR primers
Primer application
PCR* RT-PCR*
Microbial community DNA
RFLP* TGGE*
DGGE* SSCP*
Clone libraries
Cloning
Databases
DNA sequencing
Bacterial isolates
Microbial community fingerprints

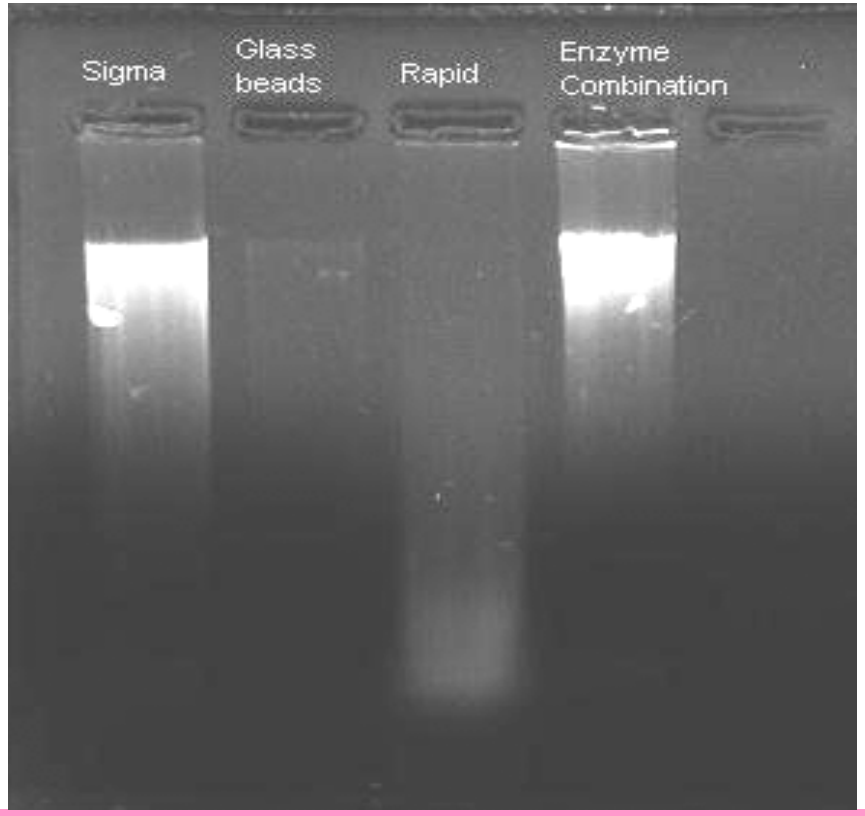
Steps involved in the study of microflora in extreme environment

- Isolation of total DNA from environment
- PCR amplification of 16S rRNA genes (rDNA)
- Construction of community profile by DNA fingerprinting techniques such as DGGE, TTGE, SSCP *etc*.
- Identification of individual species by sequencing
- Construction of reference ladder specific for healthy reactors / anaerobic digester.
- Development of culture bank for startup of healthy reactors / recovery of sour digesters

rRNA: The Ultimate Molecular Chronometers

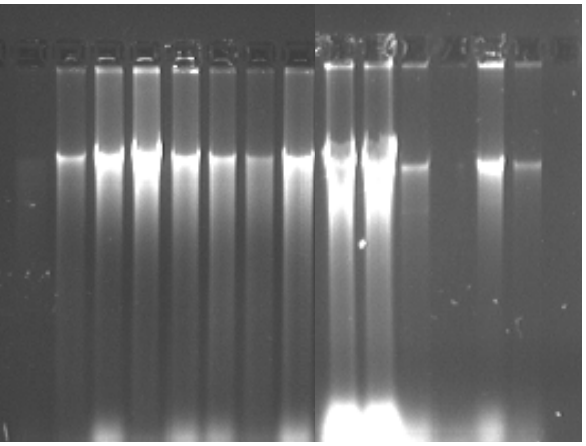
- 16S rRNA genes exhibit functional constancy
- Play important role in protein synthesis and is present in all cellular organisms.
- Most mutations in rRNA are harmful and rarely tend to occur.
- They lack lateral gene transfer.
- They have sufficient size i.e. 1500bp
- Conserved regions that can be used to design universal primers for amplification by polymerase chain reaction
- Variable regions that can be used for comparing the relatedness between the organisms for identification.
- Large database of 16S rDNA sequences for homology based identification available.

Isolation of total DNA from soil

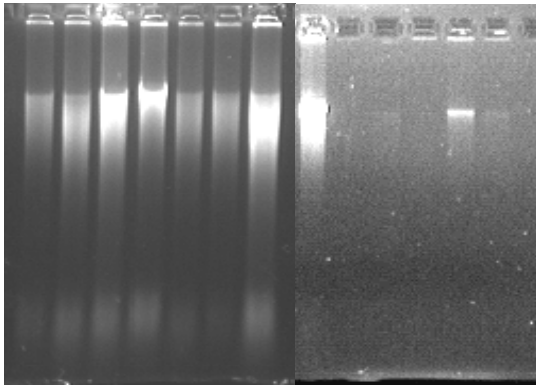


Agarose Gel Electrophoresis of Genomic DNA

Agarose Gel Electrophoresis of Genomic DNA from reference cultures

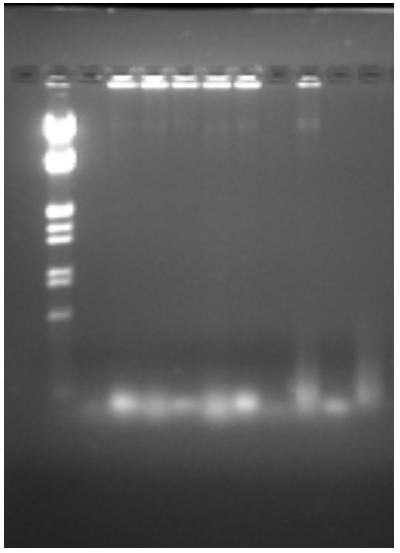


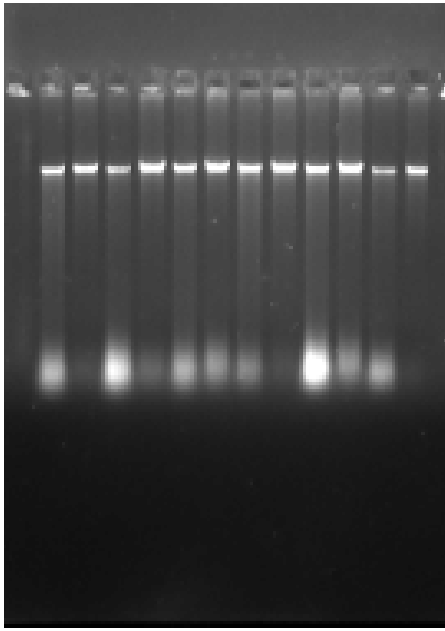
CTAB



Lysis method

Boiling



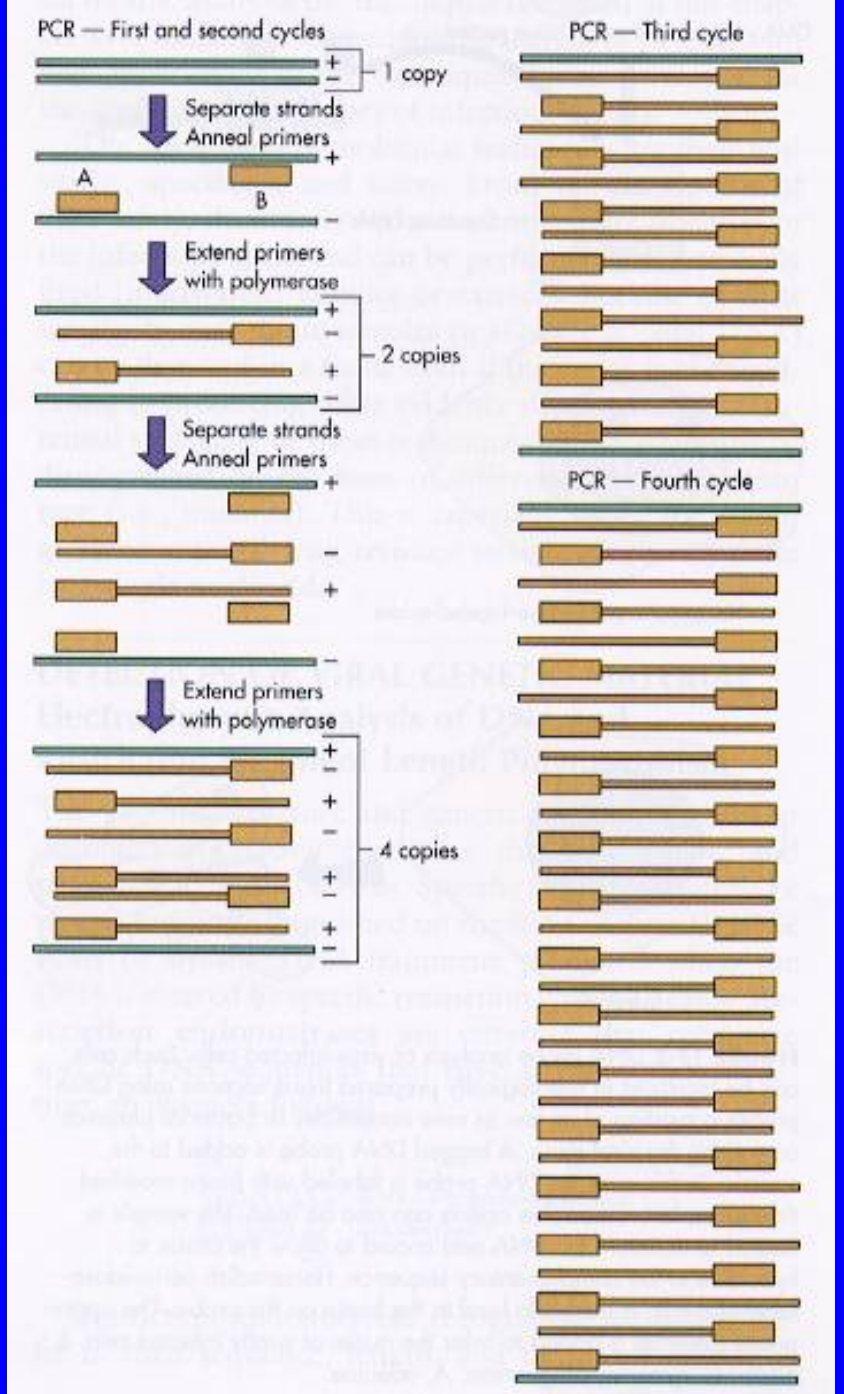


Sigma kit

PCR

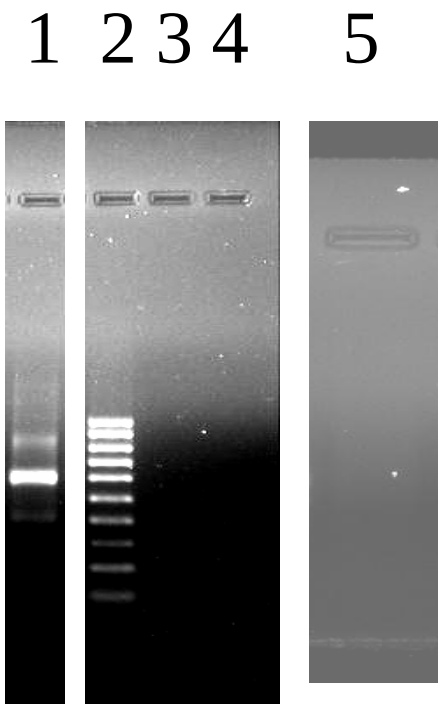
Rate of increase

2^n



RT-PCR

Agarose gel electrophoresis of Genomic DNA and PCR amplified DNA.



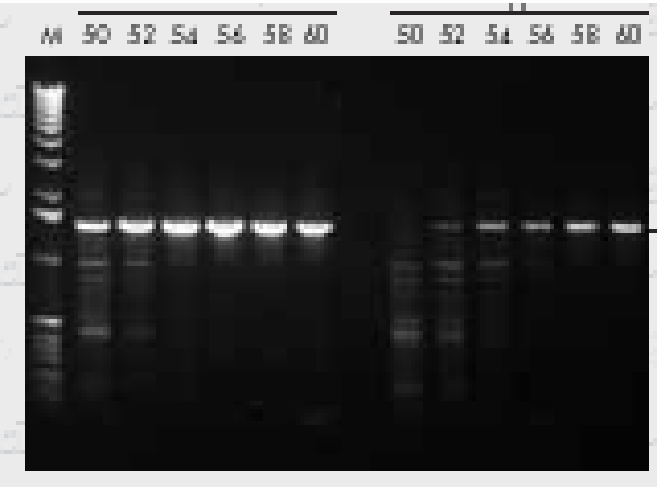
Optimization of PCR conditions for specific amplification of 16S rDNA

With the help of gradient PCR we optimized the PCR conditions for the specific samplification of 16S rDNA as follows:

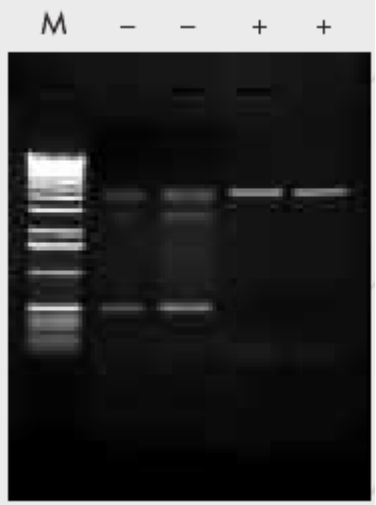
Denaturation at 94°C for 1 minute;
Annealing at 62°C for 1 minute;
Elongation at 72°C for 1 minute
Number of cycles – 30.

Since the amplified product was to be subsequently used for DGGE, GC clamp was attached to one of the forward primes used. The forward and reverse primers were such selected that the amplicon length was 600bp. This length was found to be optimum for the resolution by DGGE.

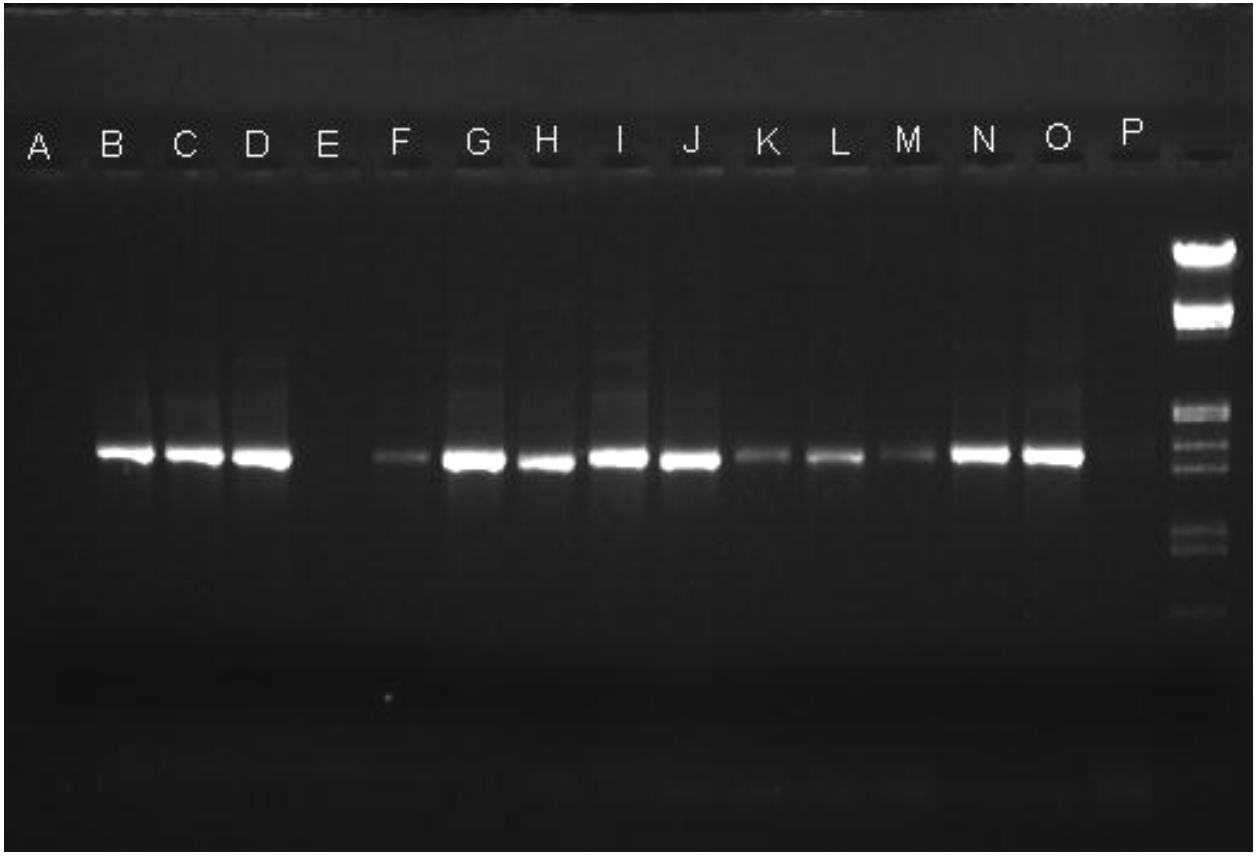
Effect of annealing temperature on PCR amplification



Effect of addition of DMSO on amplification of *GC* rich template

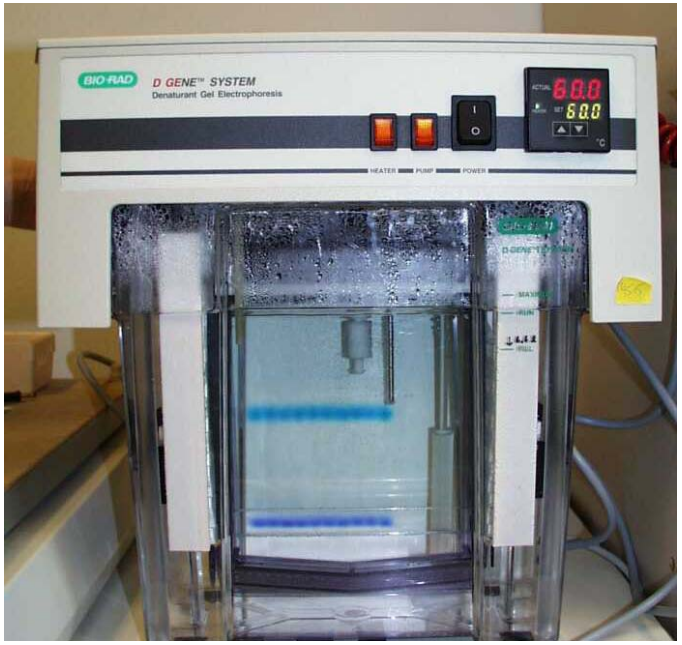


PCR



Denaturing Gradient gel electrophoresis (DGGE)

- A method to separate DNA fragments different from each other by even a single base substitution on the basis of their melting properties



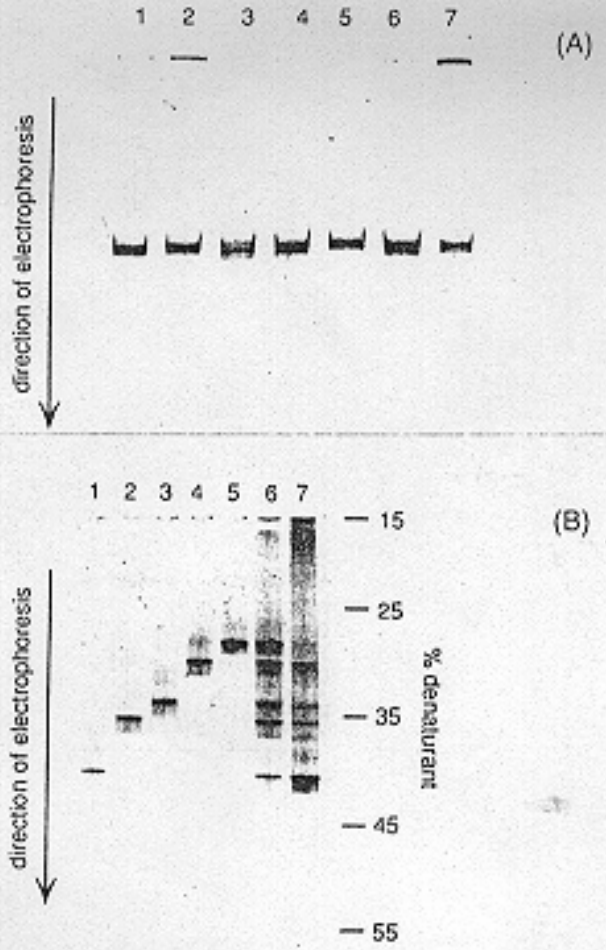


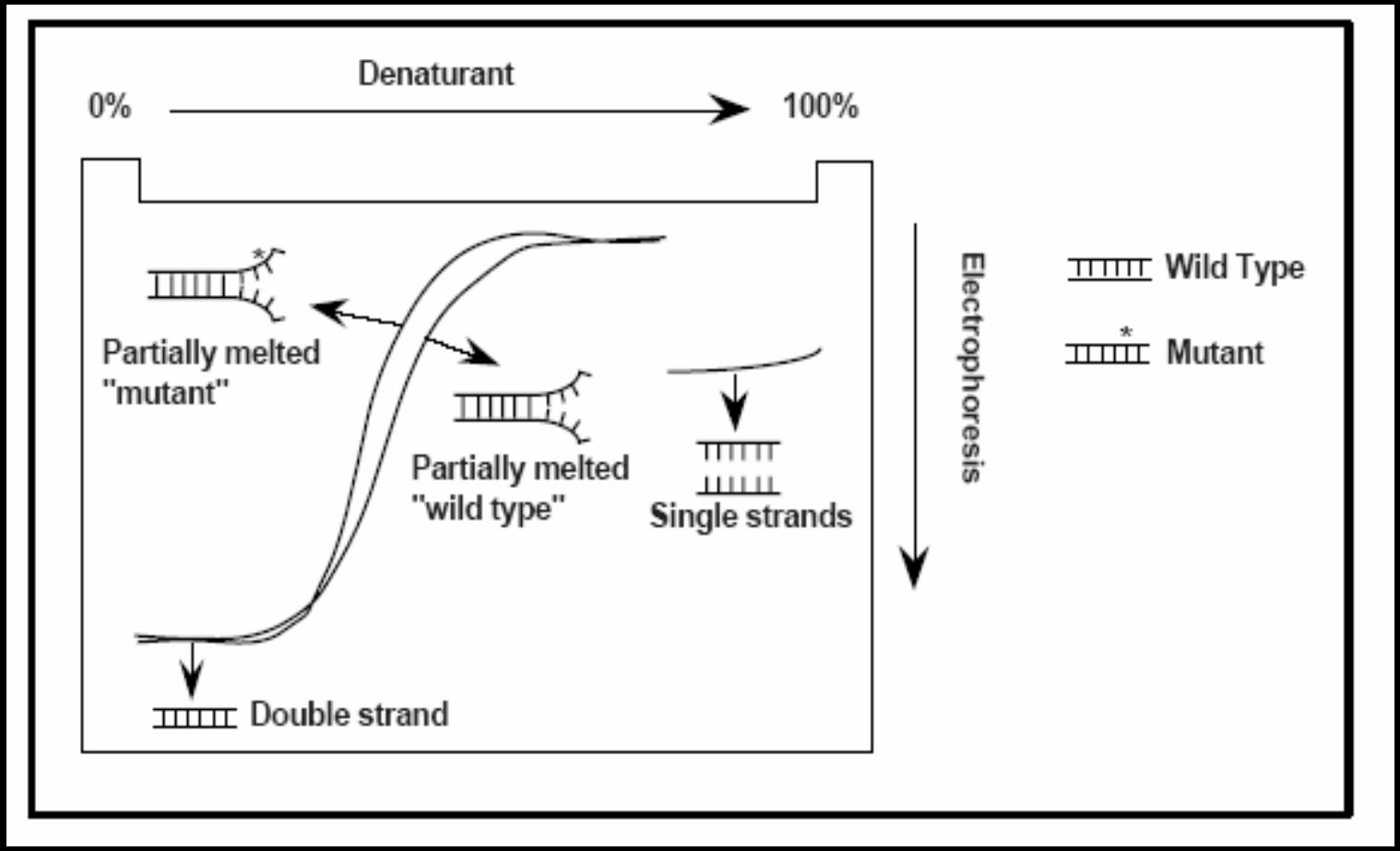
FIG. 5. Neutral polyacrylamide (A) and DGGE (B) analyses of 16S rDNA fragments of different eubacteria obtained after PCR amplification with primers 3 and 2. Both figures show negative images of ethidium bromide-stained separation patterns of *D. sapovorans* (lanes 1), *E. coli* (lanes 2), *M. chthonoplastes* (lanes 3), *T. thioparus* (lanes 4), *D. desulfuricans* (lanes 5), a mixture of these PCR products (lanes 6), and a sample obtained after enzymatic amplification of a mixture of the bacterial genomic DNAs (lanes 7).

DGGE protocol

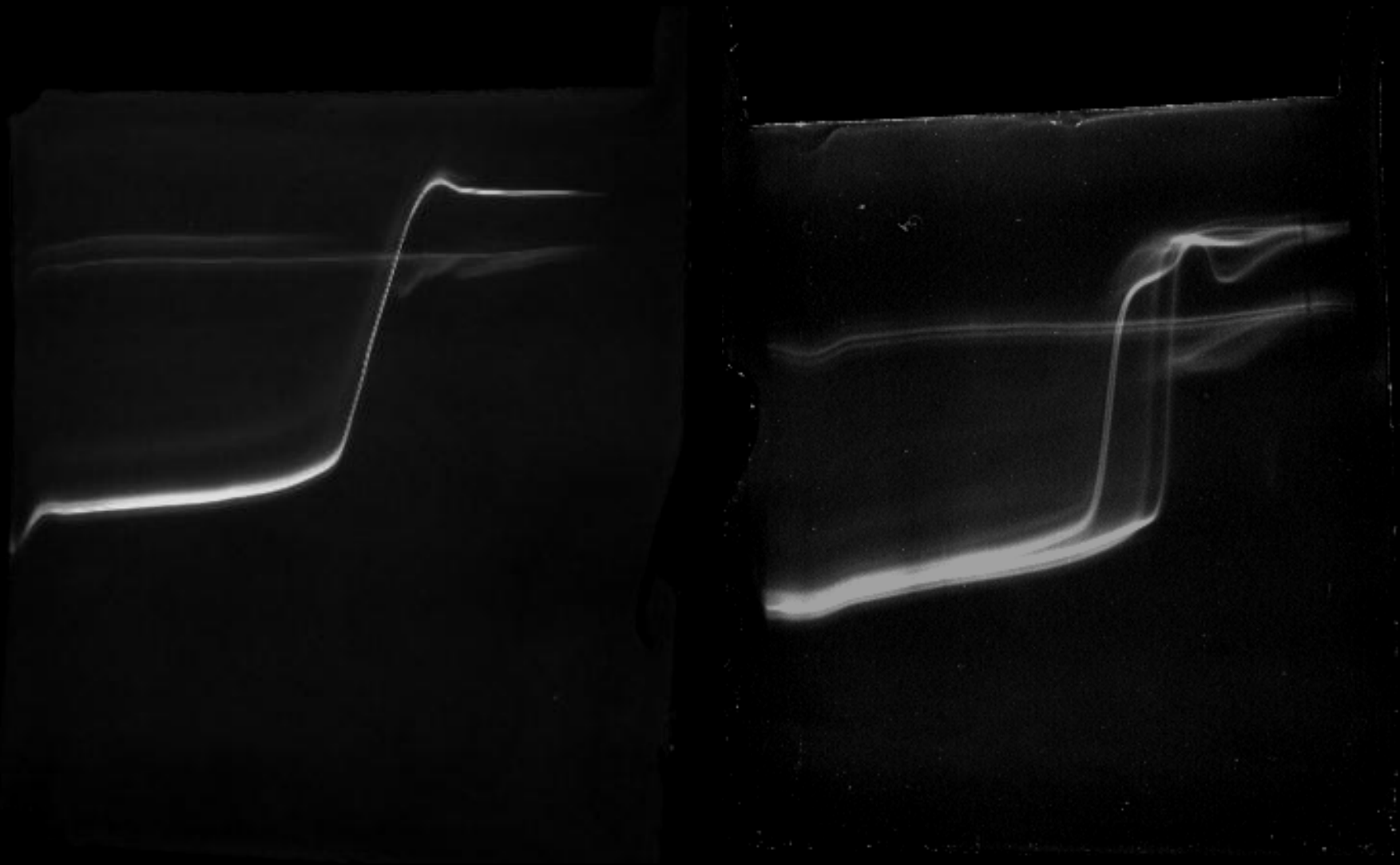
- Isolation of community DNA
- Amplification of 16S rDNA or its hypervariable region by PCR using universal primers
- Electrophoresis of the DNA fragment(s) through the linearly increasing gradient of denaturants (chemical or thermal)
- Separation of bands of the same size on the basis of their base composition
- Staining of the gels with ethidium bromide
- Visualization under UV transilluminator.

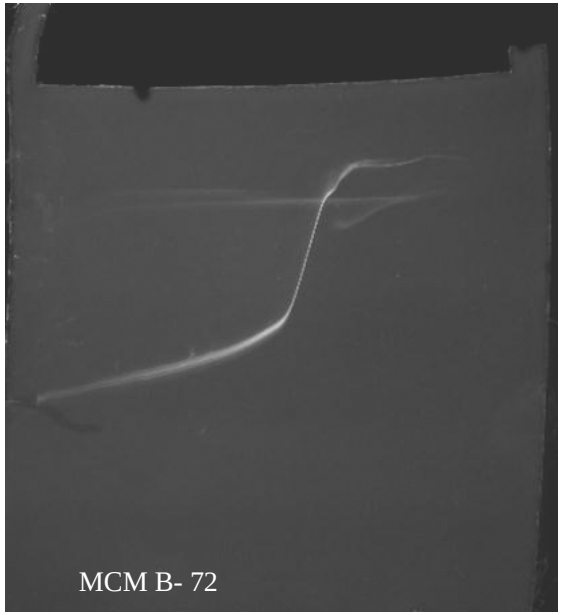
OPTIMISATION OF DENATURING GRADIENT

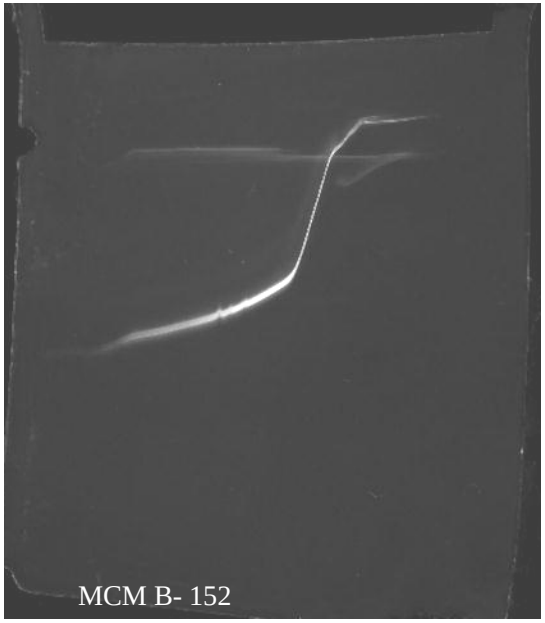
DNA melting properties in a Perpendicular denaturing gradient gel

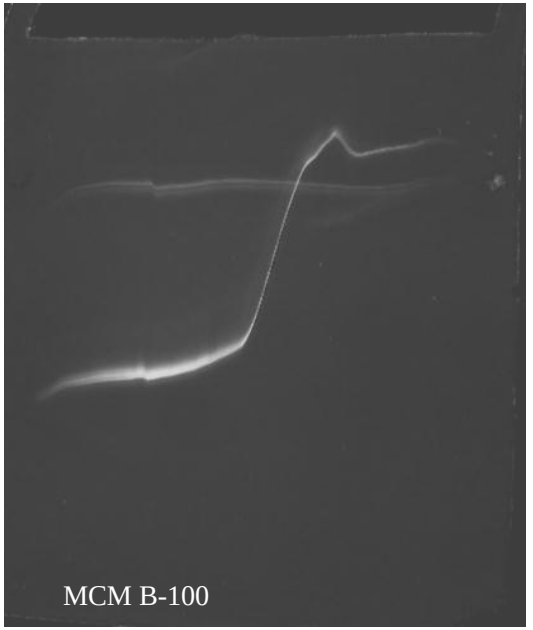


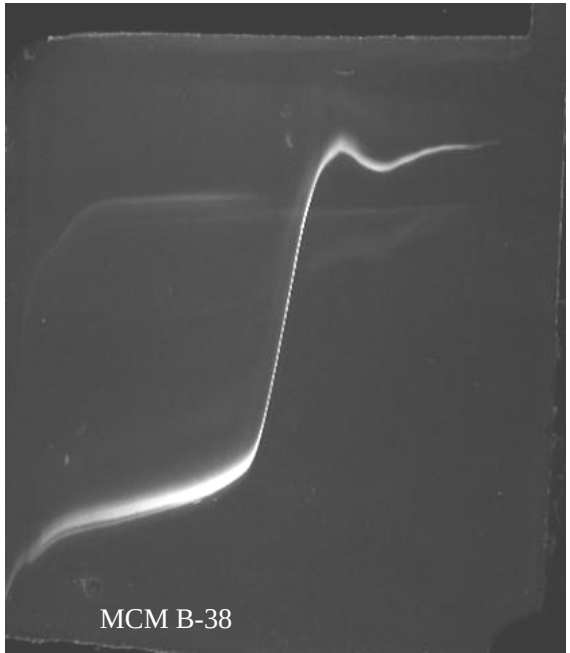
Perpendicular DGGE



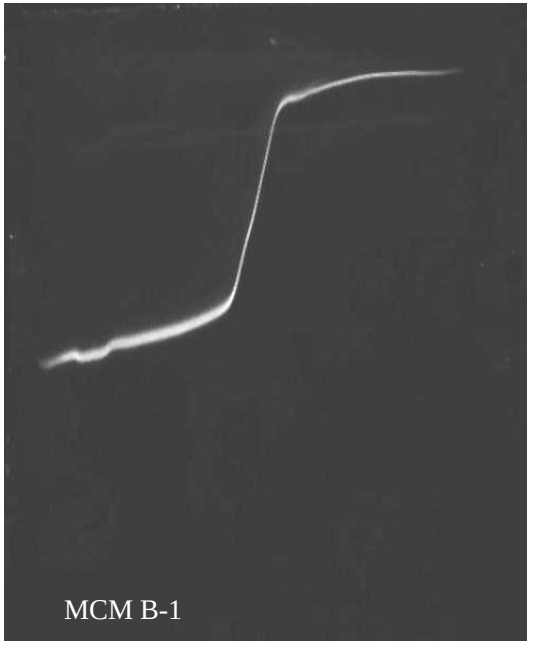

MCM B- 72


MCM B- 152

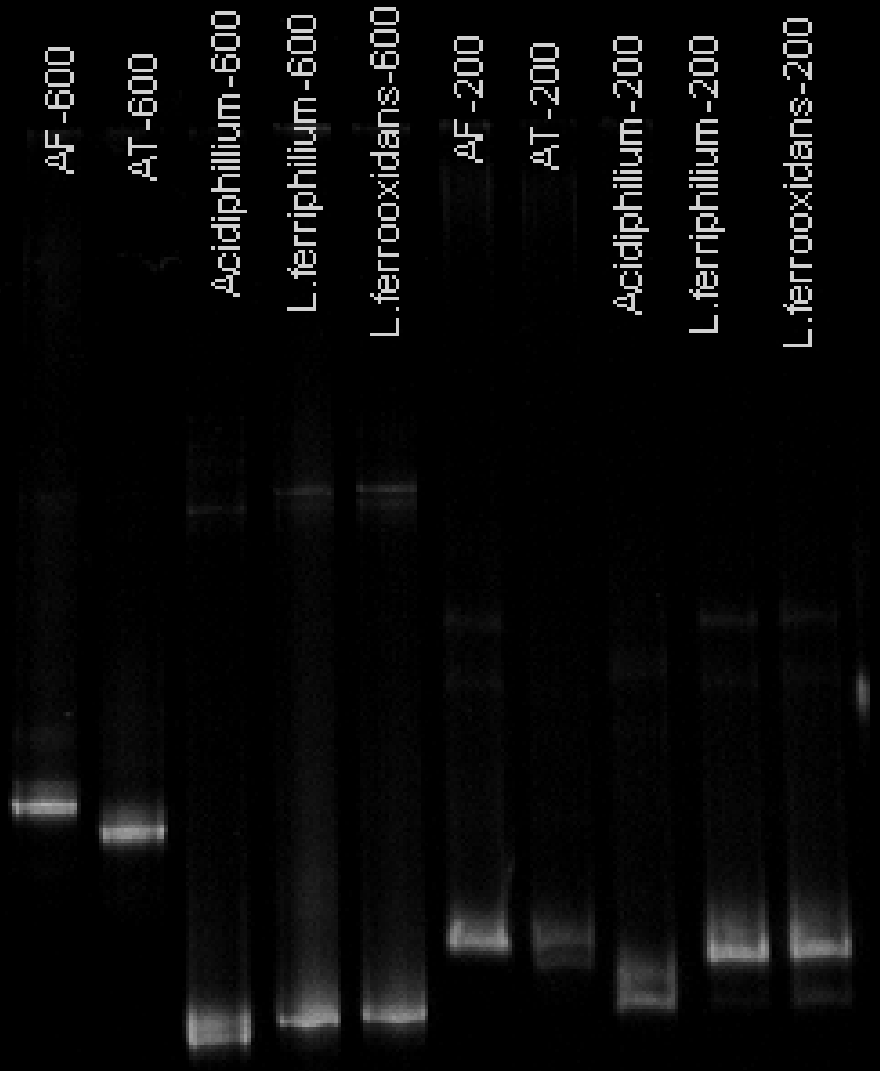

MCM B-100

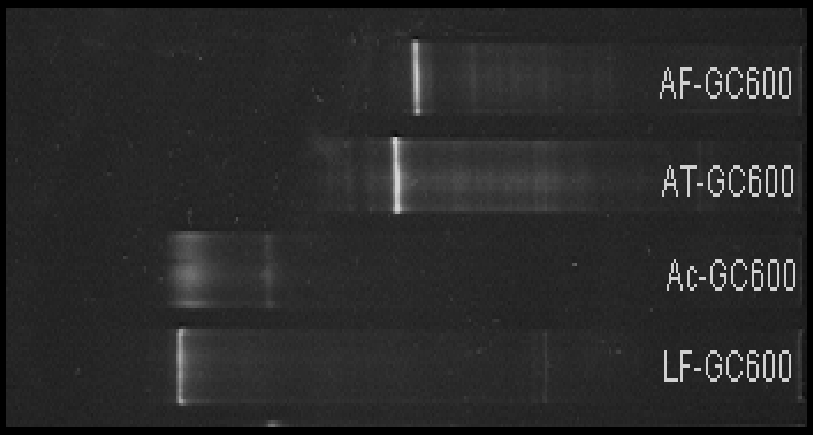

MCM B-38

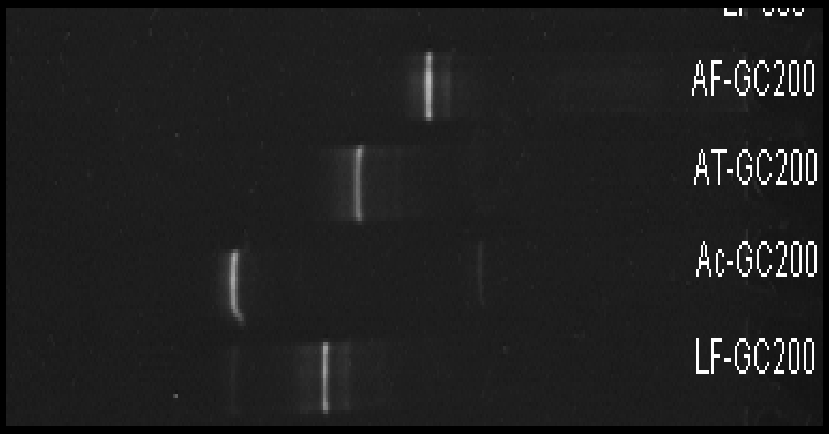

MCM B-39

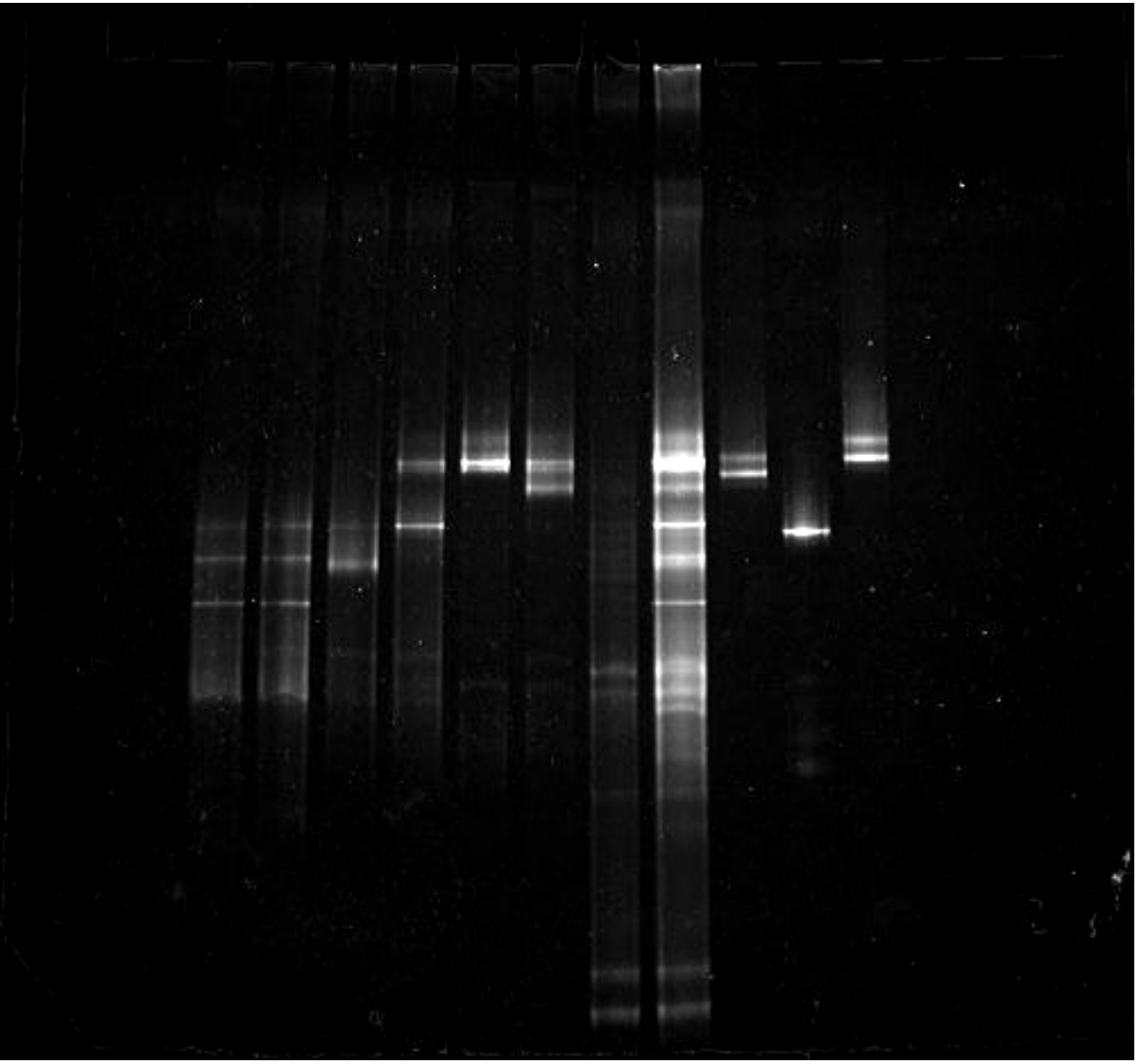

MCM B-1

Parallel DGGE




AF-GC600
AT-GC600
Ac-GC600
LF-GC600


AF-GC200
AT-GC200
Ac-GC200
LF-GC200



30%

1. *M .formicicum*
2. *M. bryantii*
3. *M. barkerii*
4. *M. bombayensis*
5. *C. butyricum*
6. *S. lacitifax*
7. Total DNA

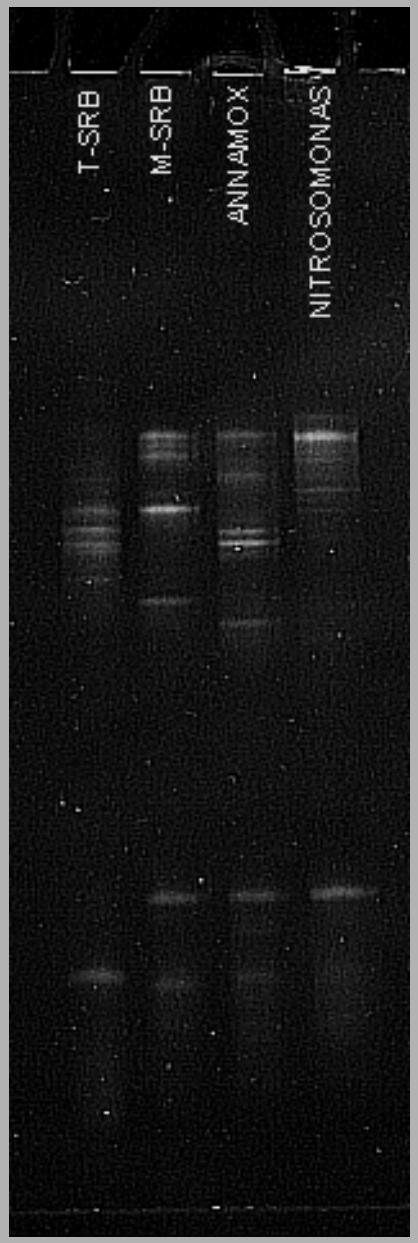
70%

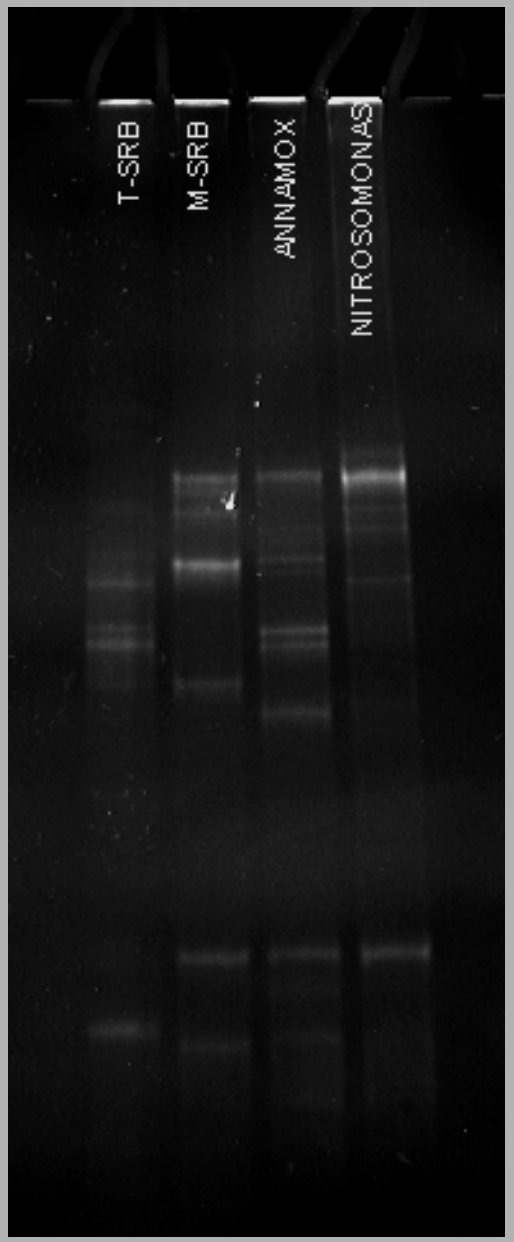
100V, 15 hrs, TAE buffer

40-60% gradient

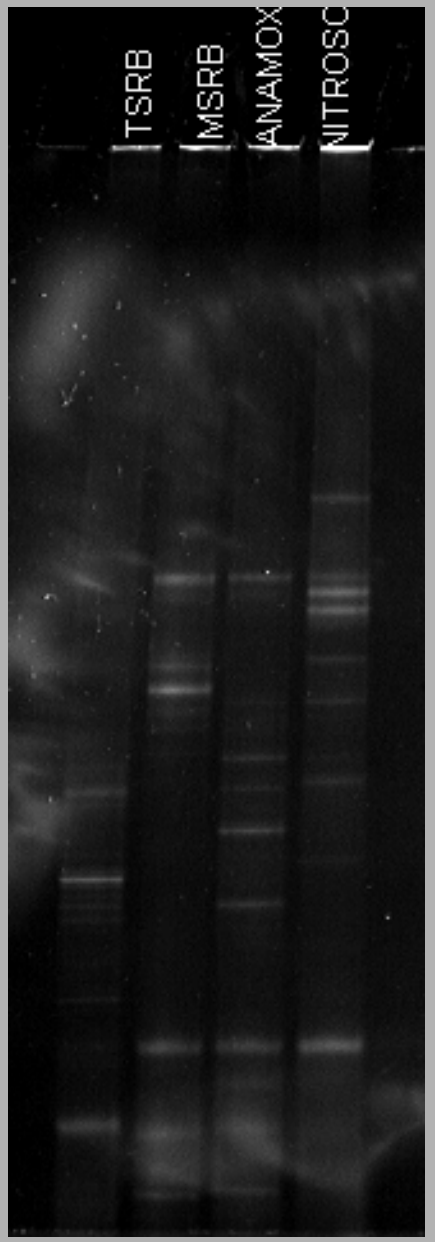
100 volts, 7.5hrs

6%acrylamide



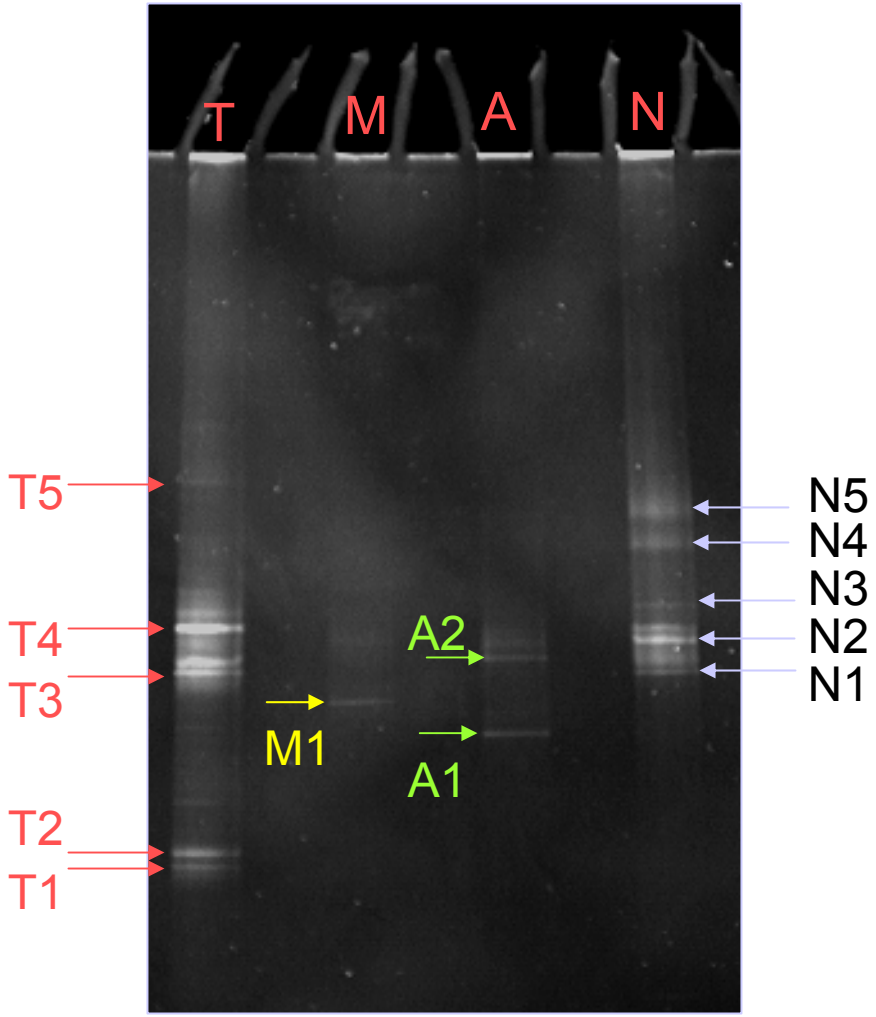


40-80%



30-60%

Investigation of microbial community structure by DGGE/sequencing



30-70% urea/formamide,
6% acrylamide, 70v -15h.

T1 - *Anaerobaculum thermoterrnum*

T2 - N.I.

T3 – N.I.

T4 - Clostridium sp.

T5 - Clostridium hongkongensis

M1 – Desulfovibrio ferophilus

A1 – Nitrospira sp.

A2- Ralstonia sp.

N1- Pseudoxanthomonas kaohsiungensis

N2- Pseudoxanthomonas daejeonensis

N3 – N.I.

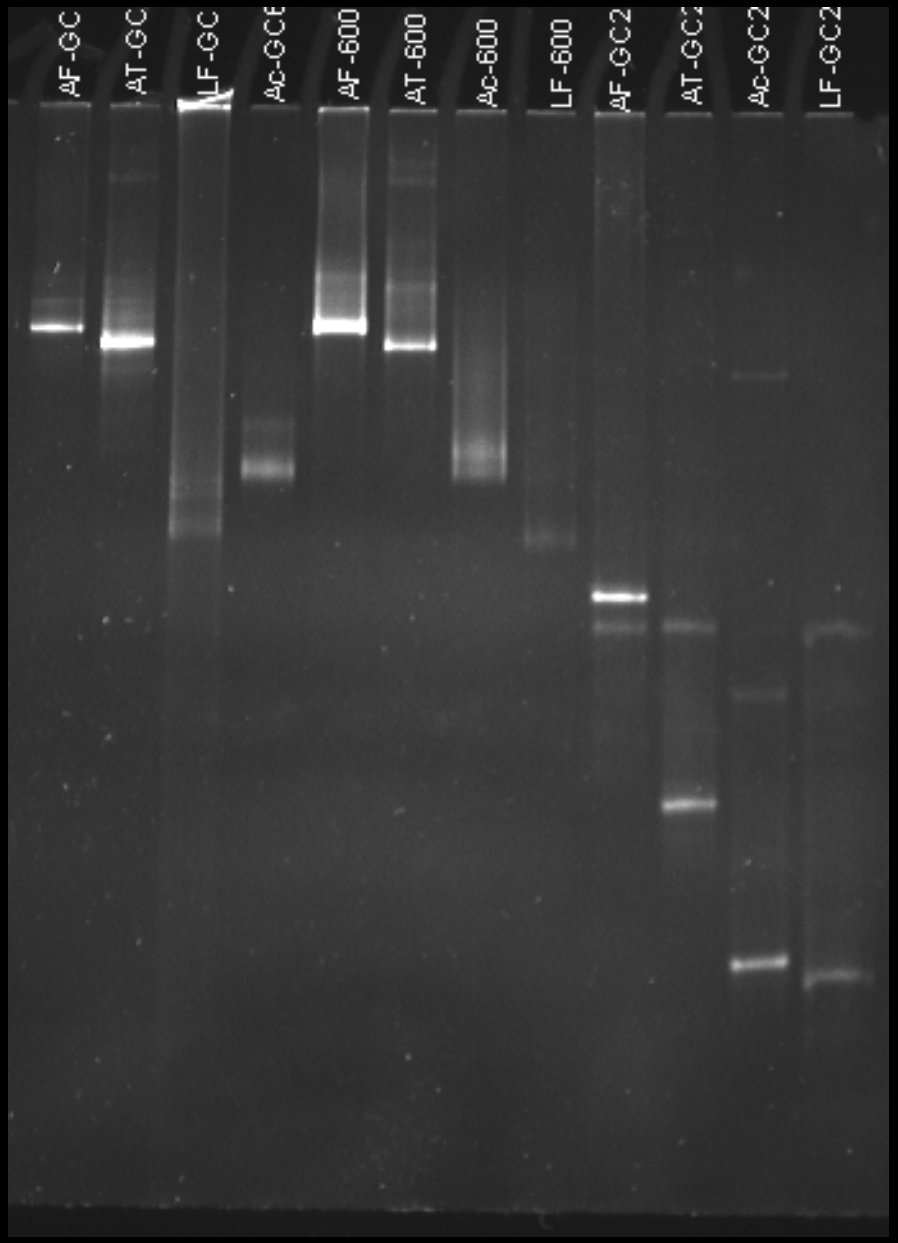
N4 – Nitrosomonas sp.

N5 – Nitrosomonas europaea

N.I. – not identifiable

T: Thermophilic SRB; M: Mesophilic SRB; A:ANAMMOX; N:Nitrifiers

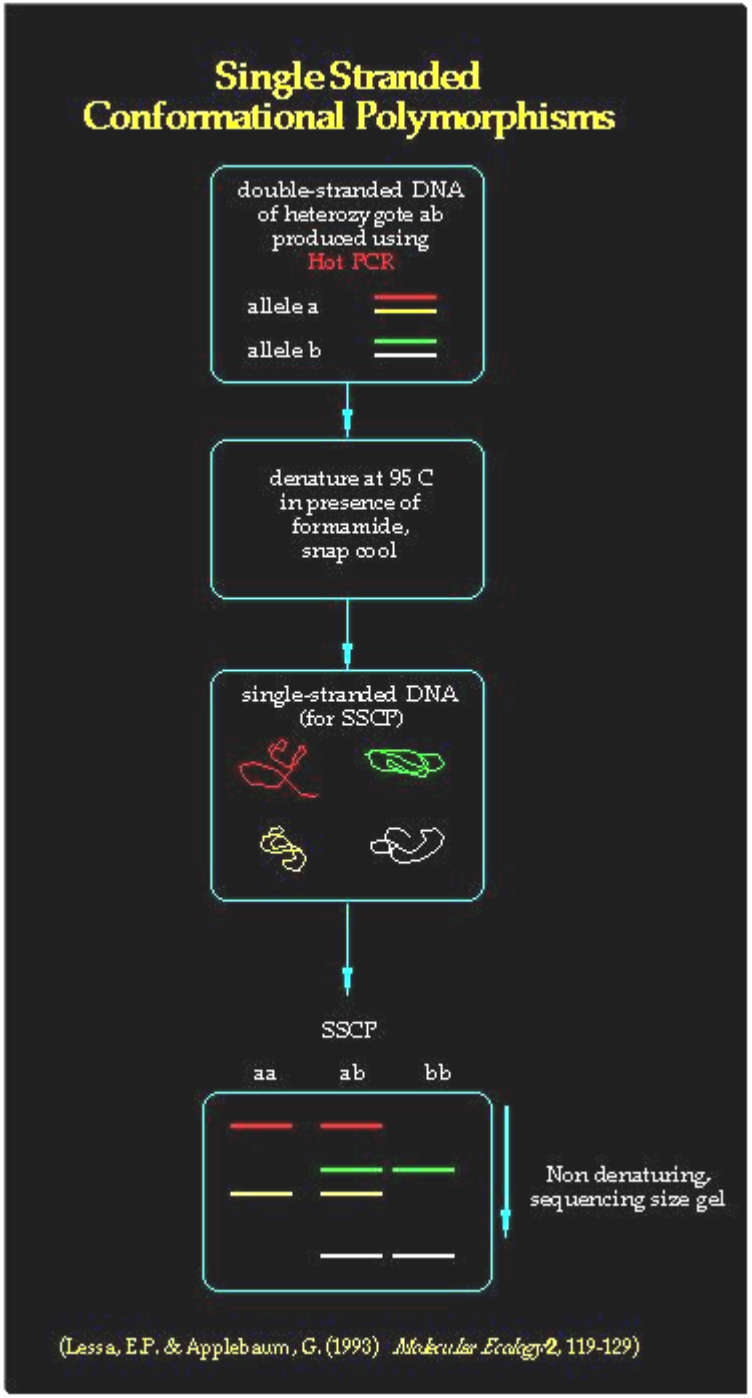
TTGE


AF-GC
AT-GC
LF-GC
Ac-GC
AF-600
AT-600
Ac-600
LF-600
AF-GC2
AT-GC2
Ac-GC2
LF-GC2

Advantages of DGGE

- Provides display of the constituents of the population in qualitative and semiquantitative way
- It is less time consuming and not laborious
- Sequencing of different clones with the same inserted DNA fragment is avoided
- Separated DNA fragments can be probed with species- or group- specific oligonucleotides
- Separated fragments can be excised, re-amplified and sequenced directly without cloning

Single Stranded Conformational Polymorphisms



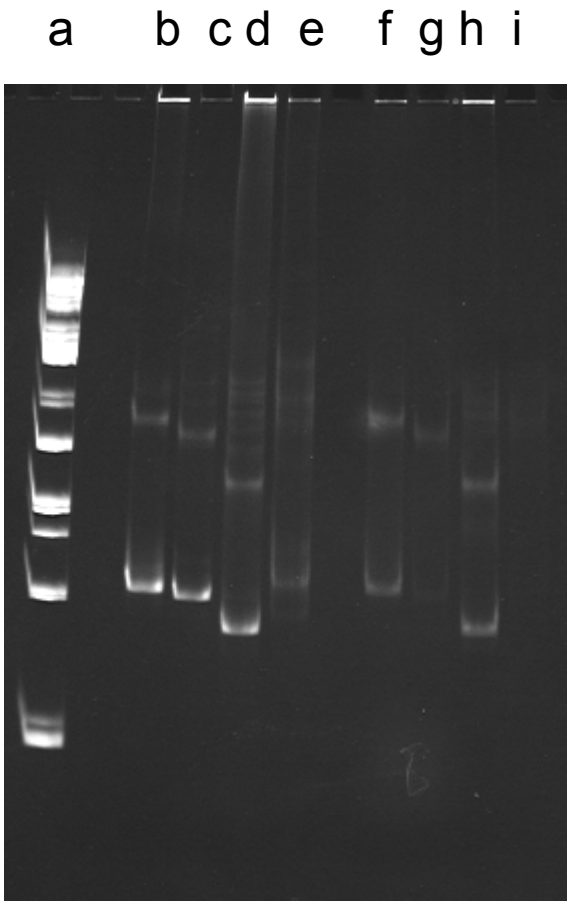
(Lessa, E.P. & Applebaum, G. (1993) *Molecular Ecology* **2**, 119-129)

Single Strand Conformational Polymorphism (SSCP)

- The electrophoretic mobility of single-strand DNA highly depends on the secondary structure (conformation) of the molecule.
- Such conformation changes significantly even if two sequences differ from each other by a single nucleotide.
- SSCP, thus, provides a method to detect sequence variation without the need to sequence DNA samples.

- DNA fragments (200-800 bp) amplified by PCR using specific 20-25 bp primers
- Separation of both strands in the presence of denaturants
- Gel-electrophoresis of single-strand DNA to obtain separation of the strands on the basis of nucleotide sequence differences
- Staining of the gel to detect nucleotide sequence variation in the form of separated bands.

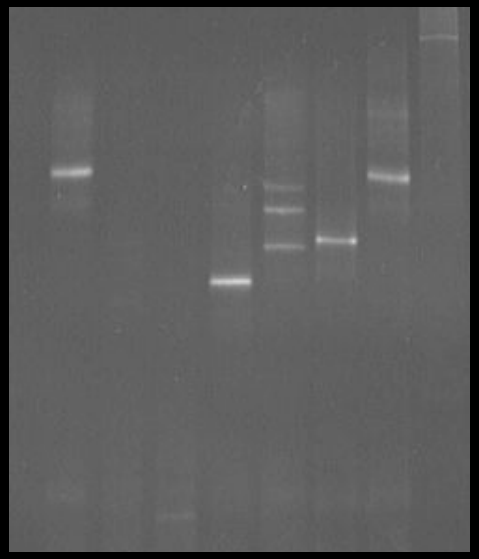
SSCP



PCR amplicons were electrophoresed on 8% SSCP gel at 300V for 3.5 h. PCR amplicons were denatured with loading dye , Lane B, AF, lane C, AT; lane D, Ac; lane E, g2-LF. PCR amplicos were digested with λ exonucl and denatured with loading dye, Lane F, AF, lane G, AT; lane H, Ac; lane I, g2-LF.

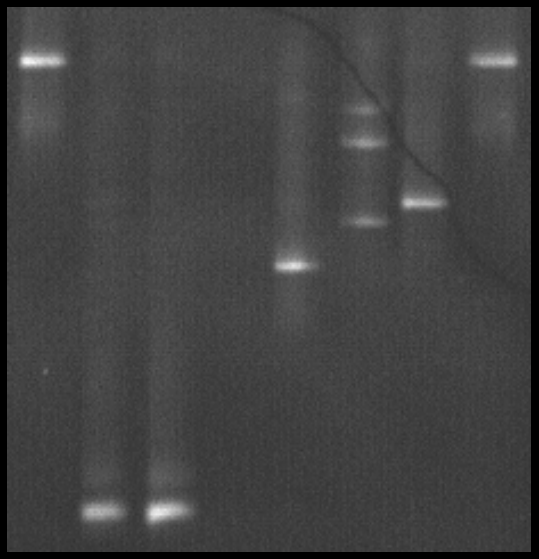
TECHNIQUES AFTER OPTIMISATION

DGGE



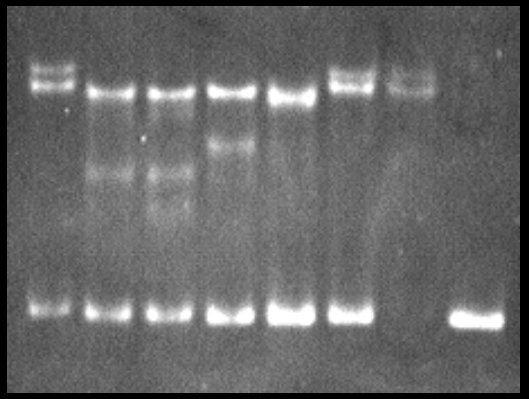
FRAGMENT SIZE:200BP
DENATURIN GRADIENT:30-50
TIME: 4.5 HR
VOLTAGE: 130V
GEL %: 6%

TTGE



FRAGMENT SIZW:200BP
RAMP RATE:0.4 DEG/HR
UREA CONC:6M
TIME:16 HR
IN & FIN TEMP:63 $ 70 DEG
VVOLTAGE 41
:GEL% 8%

SSCP



GLYCEROL%:0%
TEMPERATURE 8DEG

LOADING BUFFER
BUFFER WITH
NAOH

VOLTAGE 63V

TIME 17 HRS

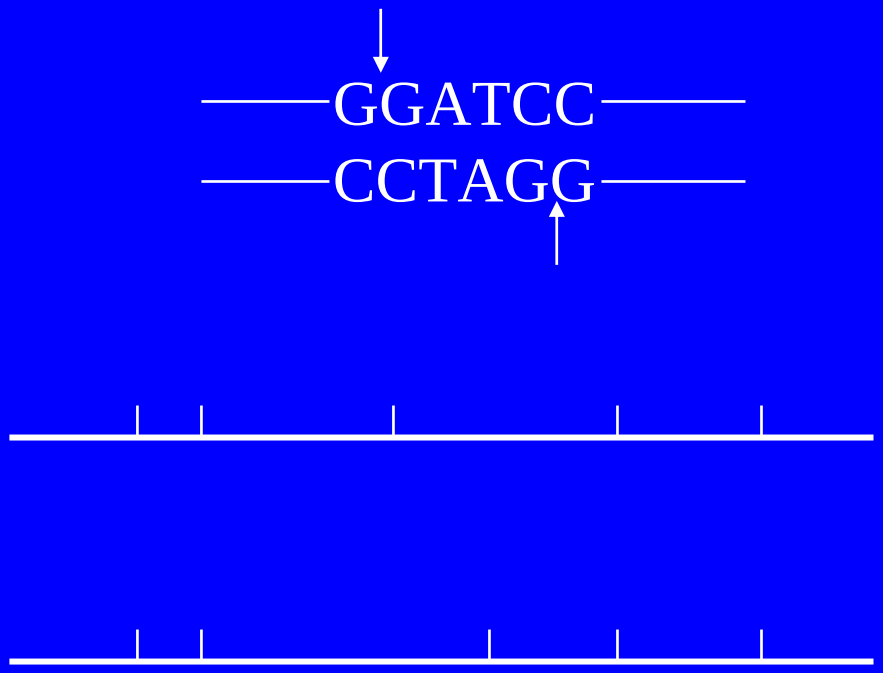
Sequencing of 16S rDNA

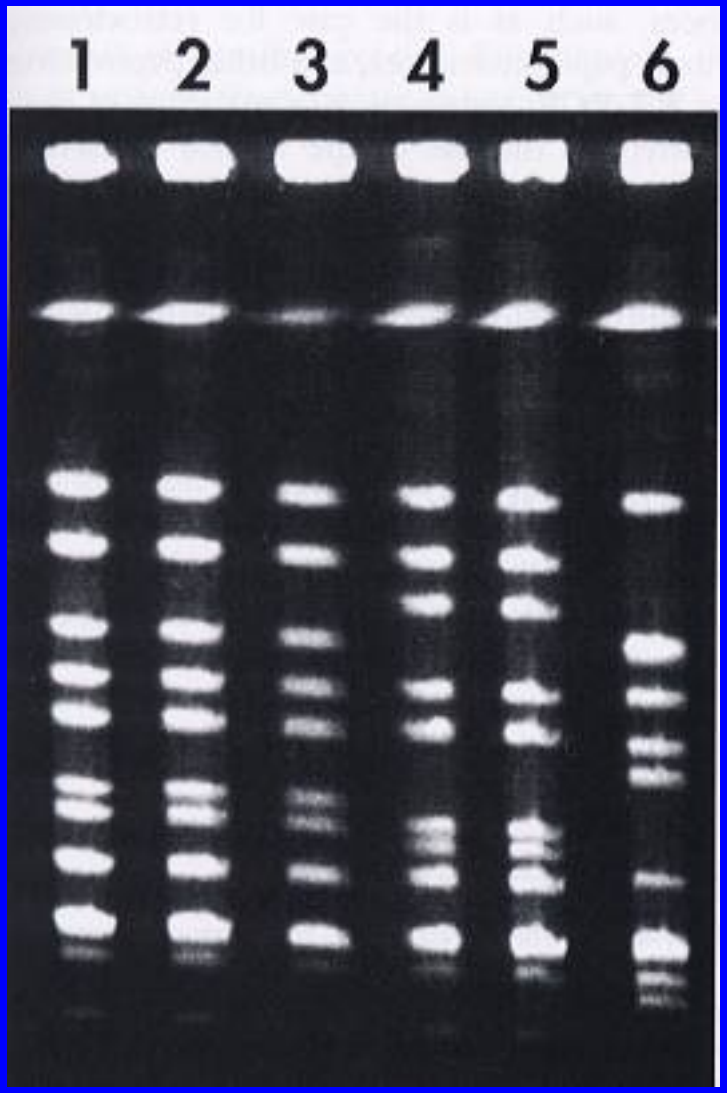
- 16S rRNA gene is amplified by a set of universal primers by Polymerase chain reaction which yields 1.5 kb long fragment.
- Amplification of exceptional sequences like the V3 region (which is about 300 bp long) is accomplished using specific primers.
- PCR amplified 16S rDNA gene cloned into a suitable vector.
- Sequencing of PCR amplified 16S rDNA.
- Sequence submitted to Blast search in the Ribosomal database.

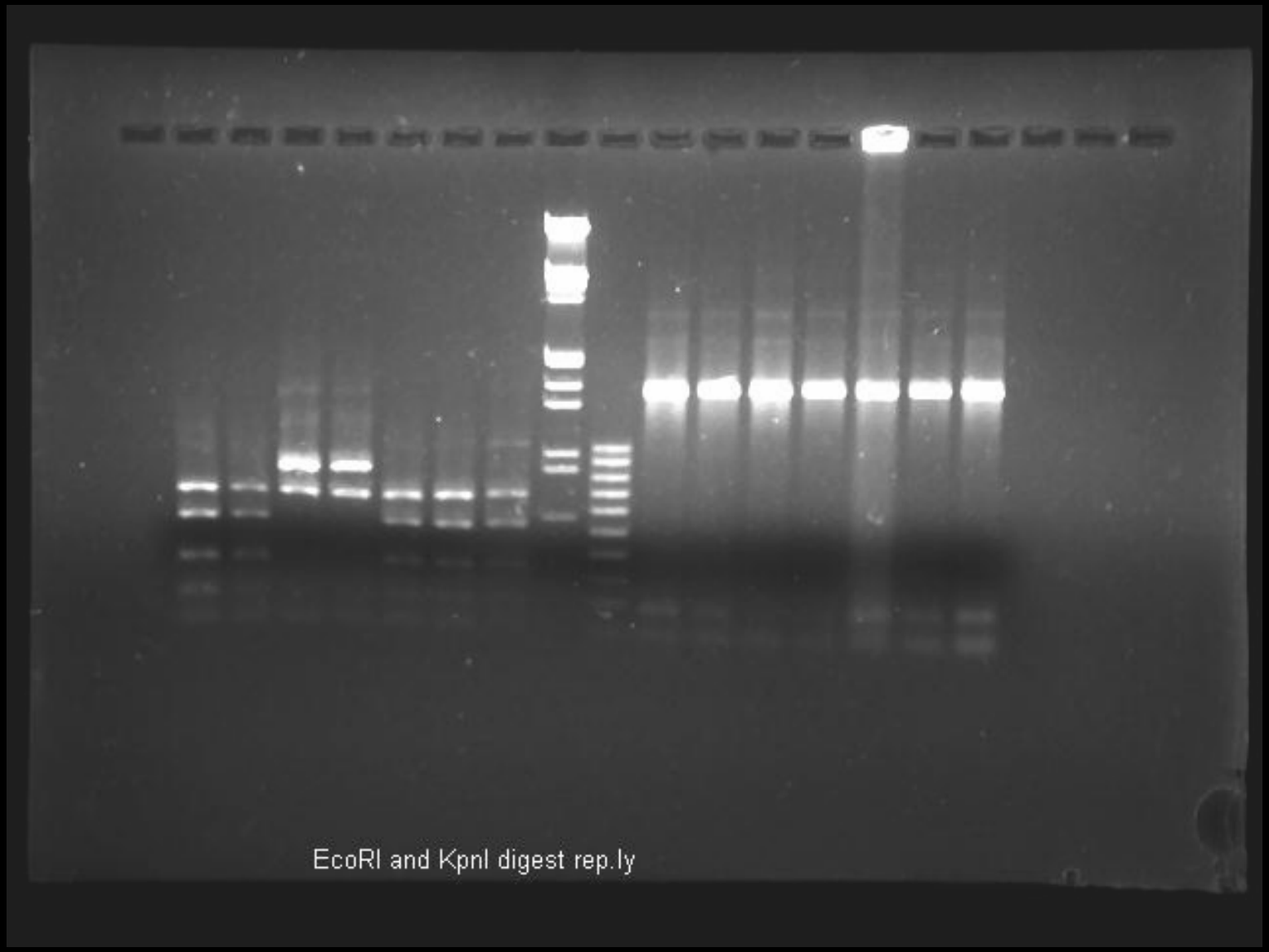
Restriction Fragment Length Polymorphism (RFLP) Pattern of 16S rDNA

- The variations in the length of DNA fragments produced by a specific restriction endonuclease from DNA(s) of two or more individuals of a species
- RFLP can be used to discriminate between closely related individuals and to establish genome maps

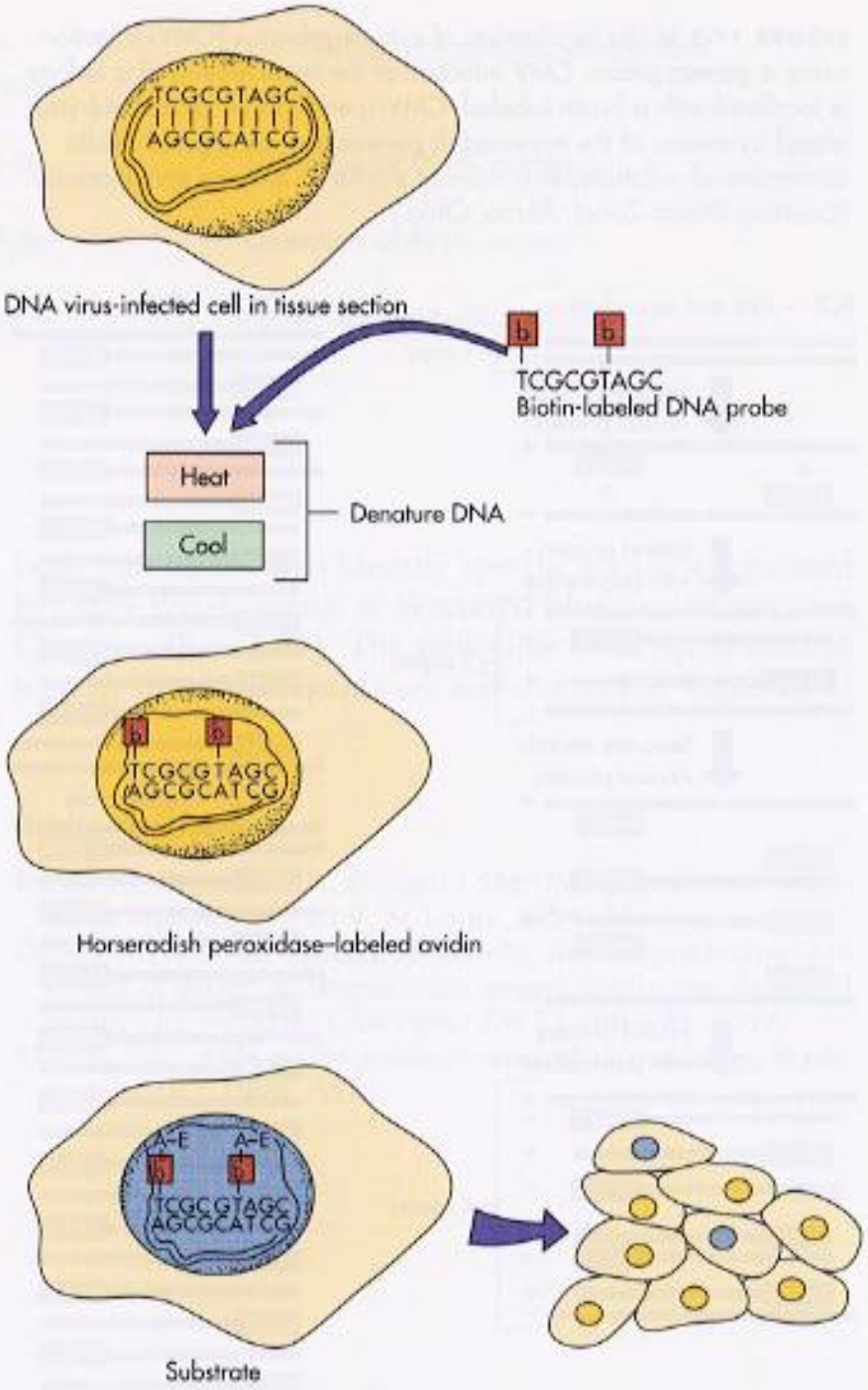
RFLP

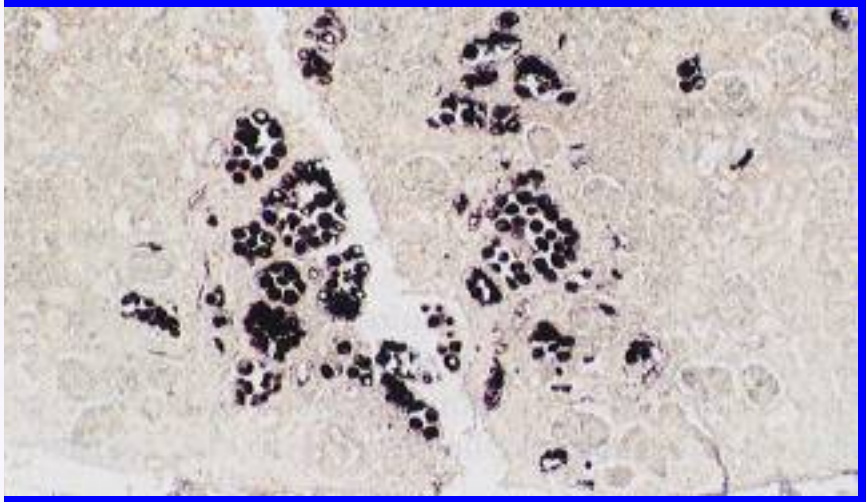





EcoRI and KpnI digest rep.ly

In situ Hybridization





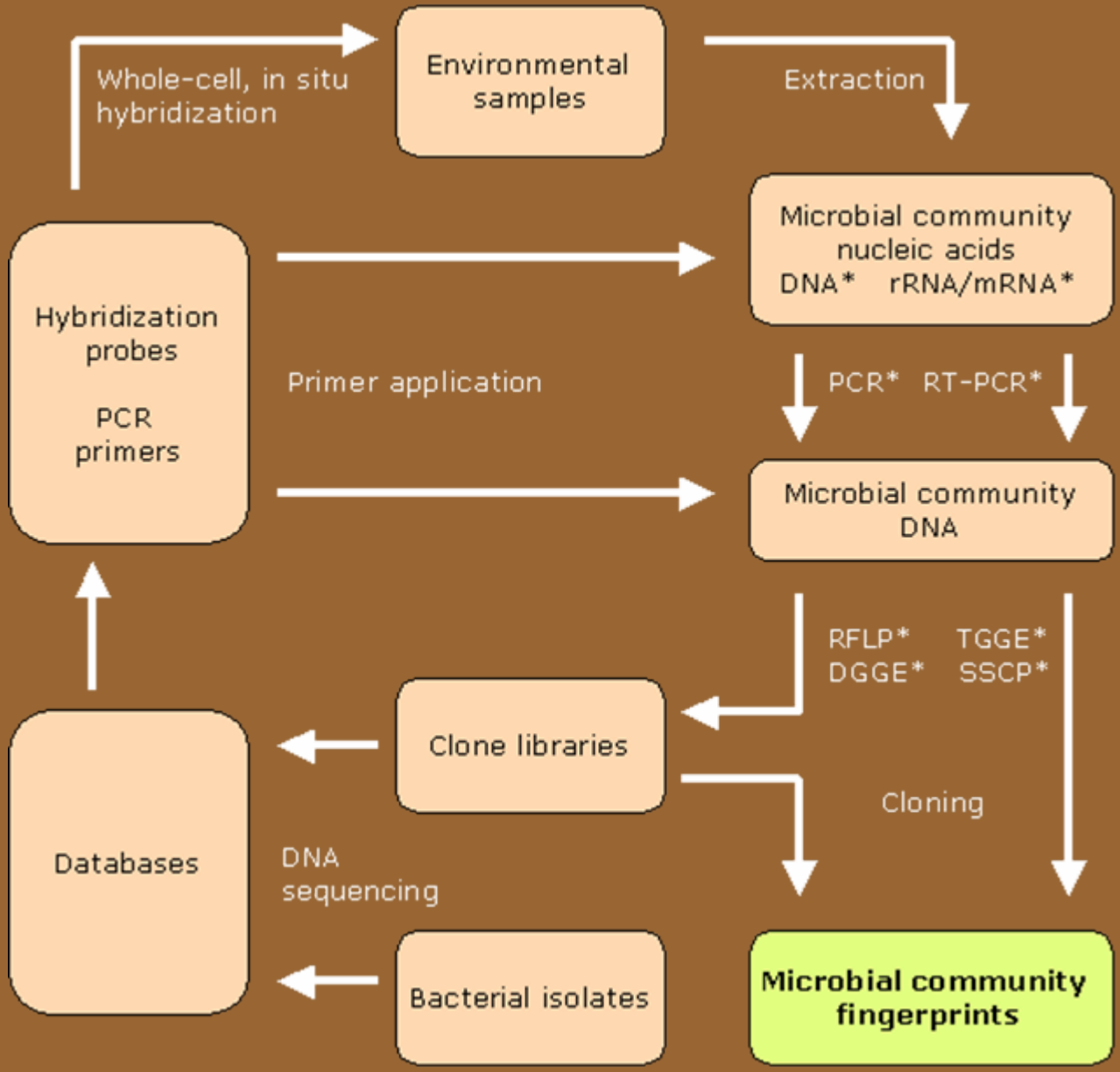
Expected outcome of the molecular diversity study

- Biodiversity conservation which would include inventorization of the entire gamut of microorganisms in a specialized ecosystem
- Isolation and identification of novel organisms with potential biotechnological application
- Standardization of molecular techniques to track these organisms in commercial processes
- Detection of these organisms in extreme environments such as industrial waste water treatment plants, thermal springs, deep sea coal beds, Antarctic ecosystems and even outer space.

Molecular Identification

- Reduce reliance on culture
- Faster
- More sensitive
- More definitive
- More discriminating
- Techniques adaptable to all pathogens

- Technically demanding
- Relatively expensive
- Can be too sensitive
- Provides no information if results are negative


Whole-cell, in situ hybridization
Environmental samples
Extraction
Microbial community nucleic acids
DNA* rRNA/mRNA*
Hybridization probes
PCR primers
Primer application
PCR* RT-PCR*
Microbial community DNA
RFLP* TGGE*
DGGE* SSCP*
Clone libraries
Cloning
Databases
DNA sequencing
Bacterial isolates
Microbial community fingerprints

Thank You!