Bioprospecting for microbes and enzymes

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Bioprospecting

Learning outcomes. Be able to…

- **Discuss the expected benefits of bioprospecting for microbes and enzymes, and some challenges involved**
- **Explain the concepts, and the hands-on methods involved in the following processes :**
  - **Isolation of a specific physiological type of microbe**
  - **Construction of a genomic or metagenomic library**
  - **Finding interesting genes in a clone library**

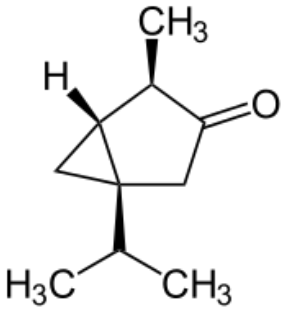
Bioprospecting – definition & history

- **Bioprospecting:** the search for organisms or their components for commercial applications

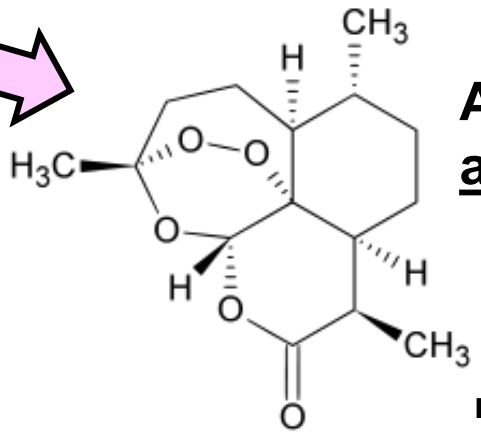
- Began with search for medicinal chemicals in plants; this aspect is still going strong…

strangerandstranger.com

organicheirloomplants.com



Thujone: antihelminthic



Artemisinin: antimalarial

microbiological significance

Bioprospecting: cells / enzymes / metabolites

- **Bioprospecting for whole microbial cells**

  → as products, as catalysts, as hosts

focus of this lecture

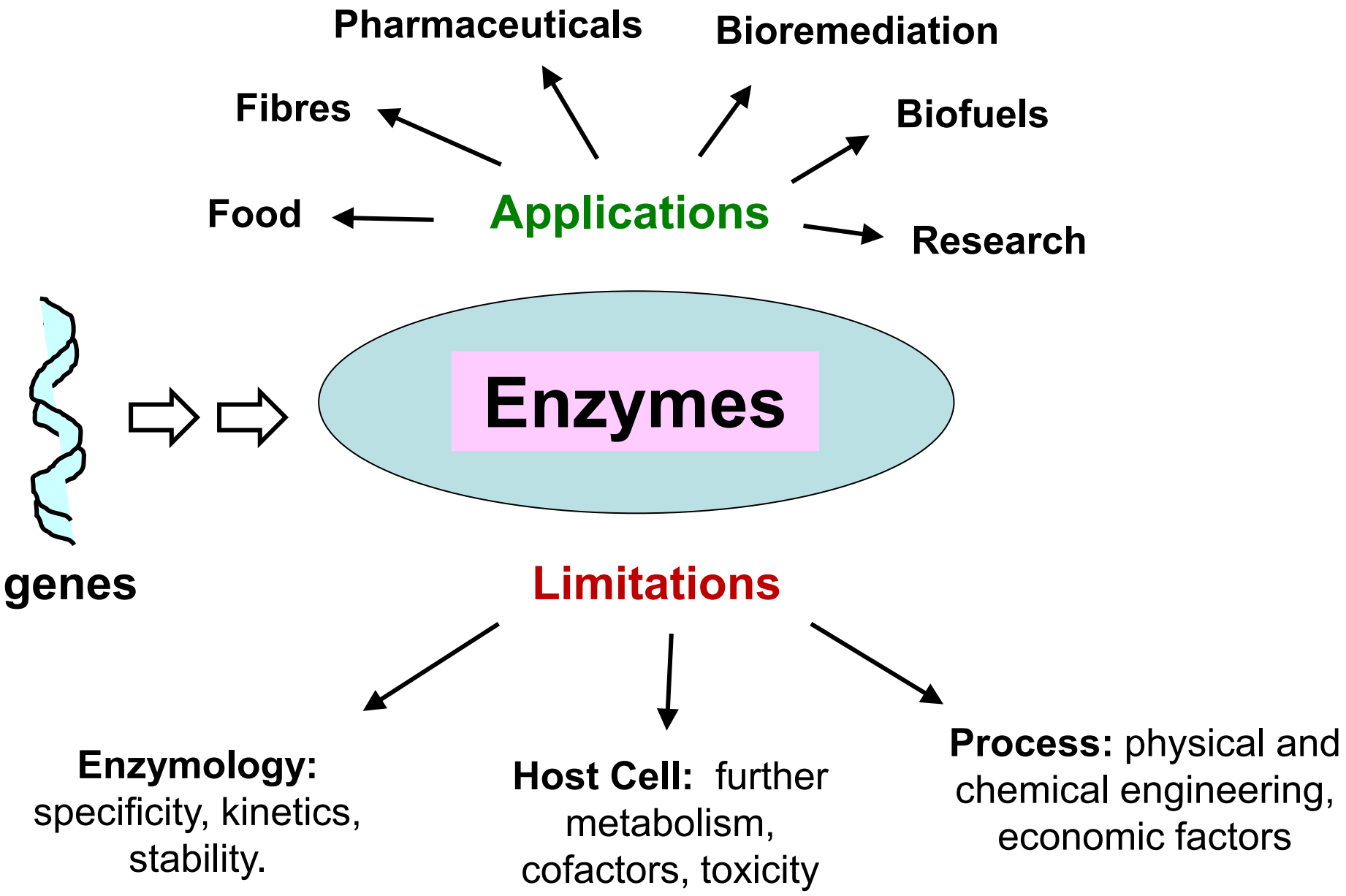
- **Bioprospecting for genes and enzymes**

  →fragments, whole genes, gene clusters

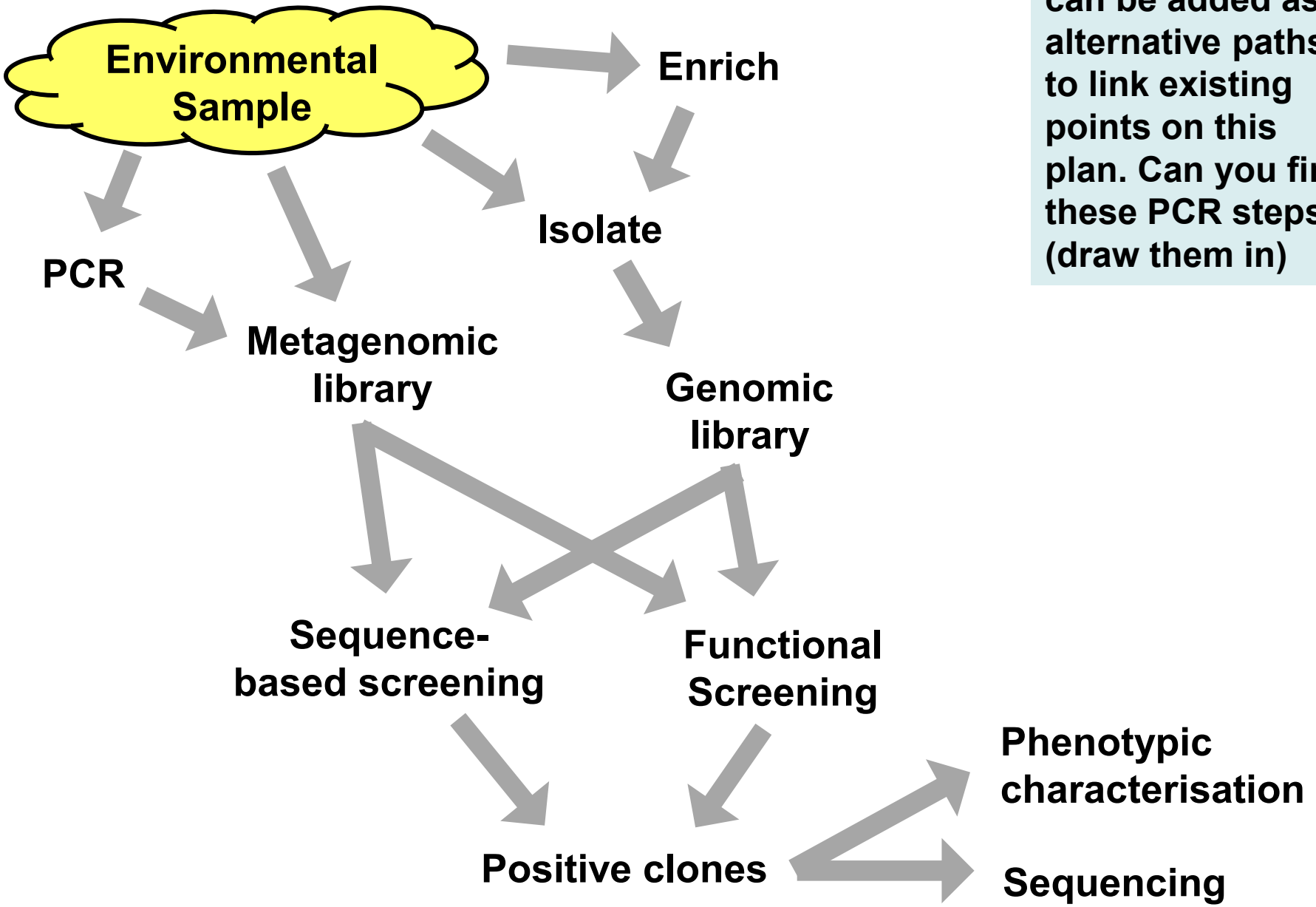
- **Bioprospecting for metabolites (small molecules)**

  → bioactive agents, usu. pharmaceuticals

Importance of ENZYMES in biotechnology



Bioprospecting: overall scheme

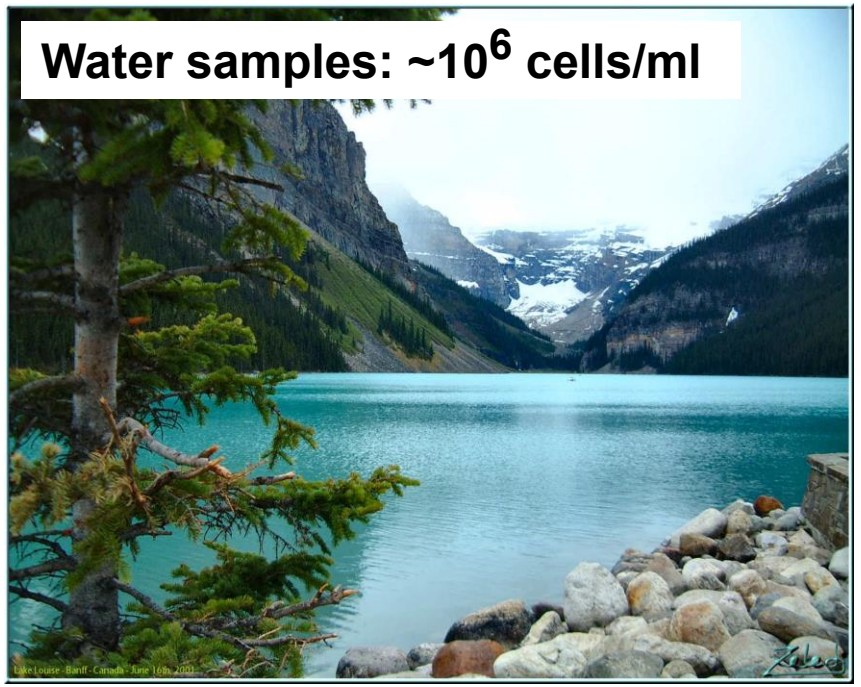


Two additional useful PCR steps can be added as alternative paths to link existing points on this plan. Can you find these PCR steps? (draw them in)

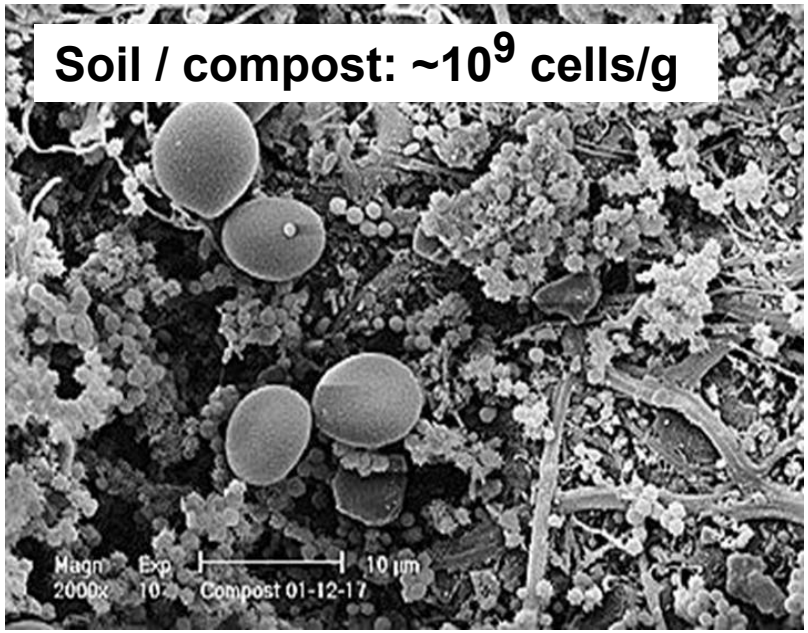
Sampling strategies

- Strategy is guided by :
  - desired traits of enzyme,
  - expected rarity of enzyme

- Other things to consider:
  - Terrestrial / aquatic ?
  - Animal / plant- associated?
  - Sample size?
  - How many samples?



imaginetravel.com.au

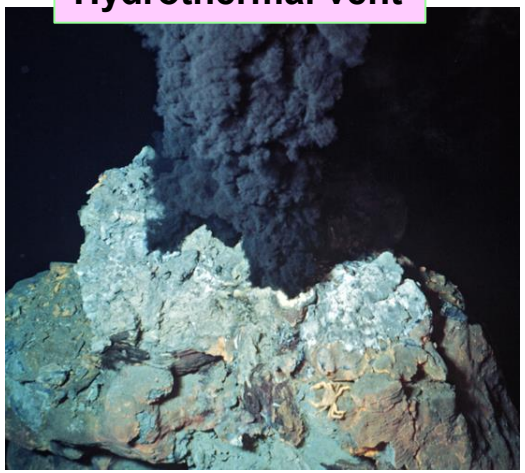


forgreenies.com

Sampling strategies - example

- **Which environments would be good to sample in the case of THERMOSTABLE POLYMERASES?**
  - **need a hot environment**
  - **…. but not so hot that it kills all microbes**
  - **need nutrients, energy, electron acceptors**
  - **other conditions (pH, toxins) cannot be too harsh**

Hydrothermal vent



whoi.edu/main/topic/hydrothermal-vents

Hot spring and geyser

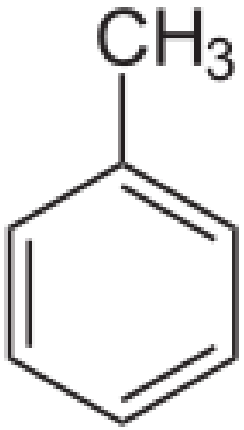
http://37.media.tumblr.com/tumblr_m66kytttL31rxqcoko1_500.jpg

Volcanic soil

http://kids.britannica.com/comptons/art-67266

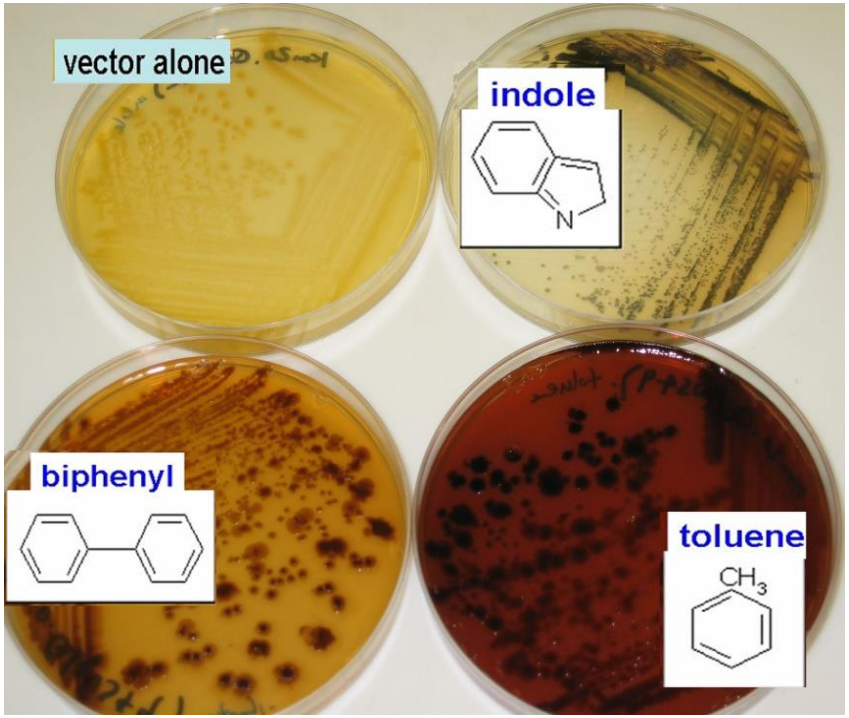
Enrichment & isolation: first principles

- Step 1. What enzyme activity are we seeking?
- Step 2. Which type of microbe has this activity?
- Step 3. How can we enrich this type of microbe? (what selective forces can we apply ?)



Example

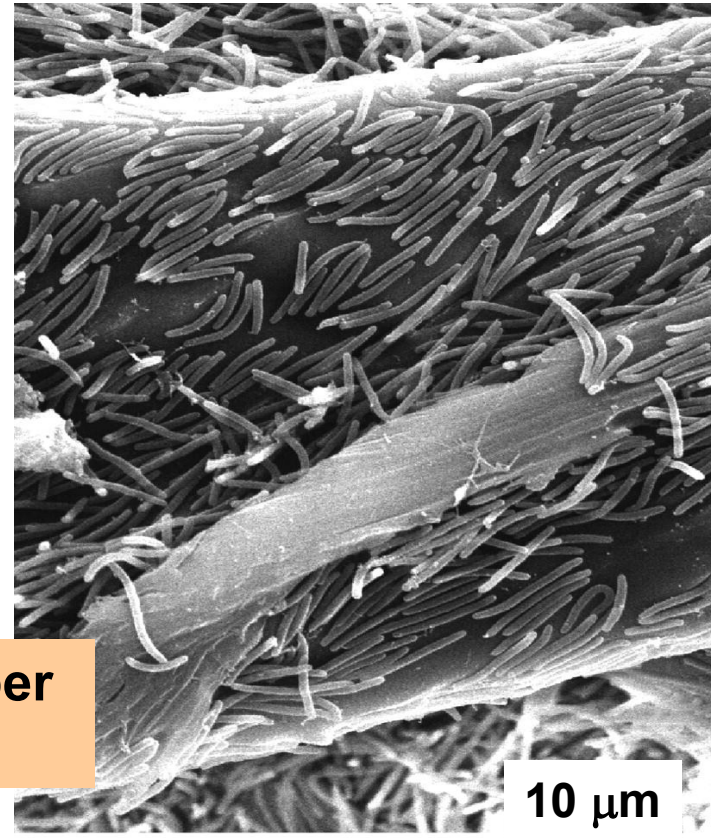
- Seeking: toluene dioxygenase enzyme
- Found in: bacteria that grow on toluene
- Enrich: minimal medium, toluene as C source
- Alternative selections or detection methods?



Detection of cloned toluene dioxygenase genes in *Pseudomonas* host via chromogenic screen

Enrichment & isolation: other considerations

- **Choice of INOCULUM, MEDIUM, CONDITIONS are crucial.**
- **e.g. with cellulose as substrate, you will get different types of microbial isolates depending on :**
  - **aerobic or anaerobic**
  - **the pH of the medium**
  - **the inoculum source**
  - **direct isolation or enrichment**



Colonisation of a cellulose fiber by *Cytophaga* bacteria

http://aem.asm.org/content/73/11/3536/F1.expansion.html

Enrichment & isolation

example: cellulose-degraders

Soil sample, acid pH, aerobic, direct isolation

Rhizosphere sample, neutral pH, aerobic, enrich

C source is the same in all cases !

All cellulose degraders

Aerobic Fungi

Aerobic G-ve bacteria

Aerobic G+ve bacteria

Anaerobic G+ve bacteria

Other strategies to select the same groups?

Faecal sample, neutral pH, anaerobic, enrich

Soil sample, neutral pH, aerobic, direct isolation

Enrichment & isolation: limitations

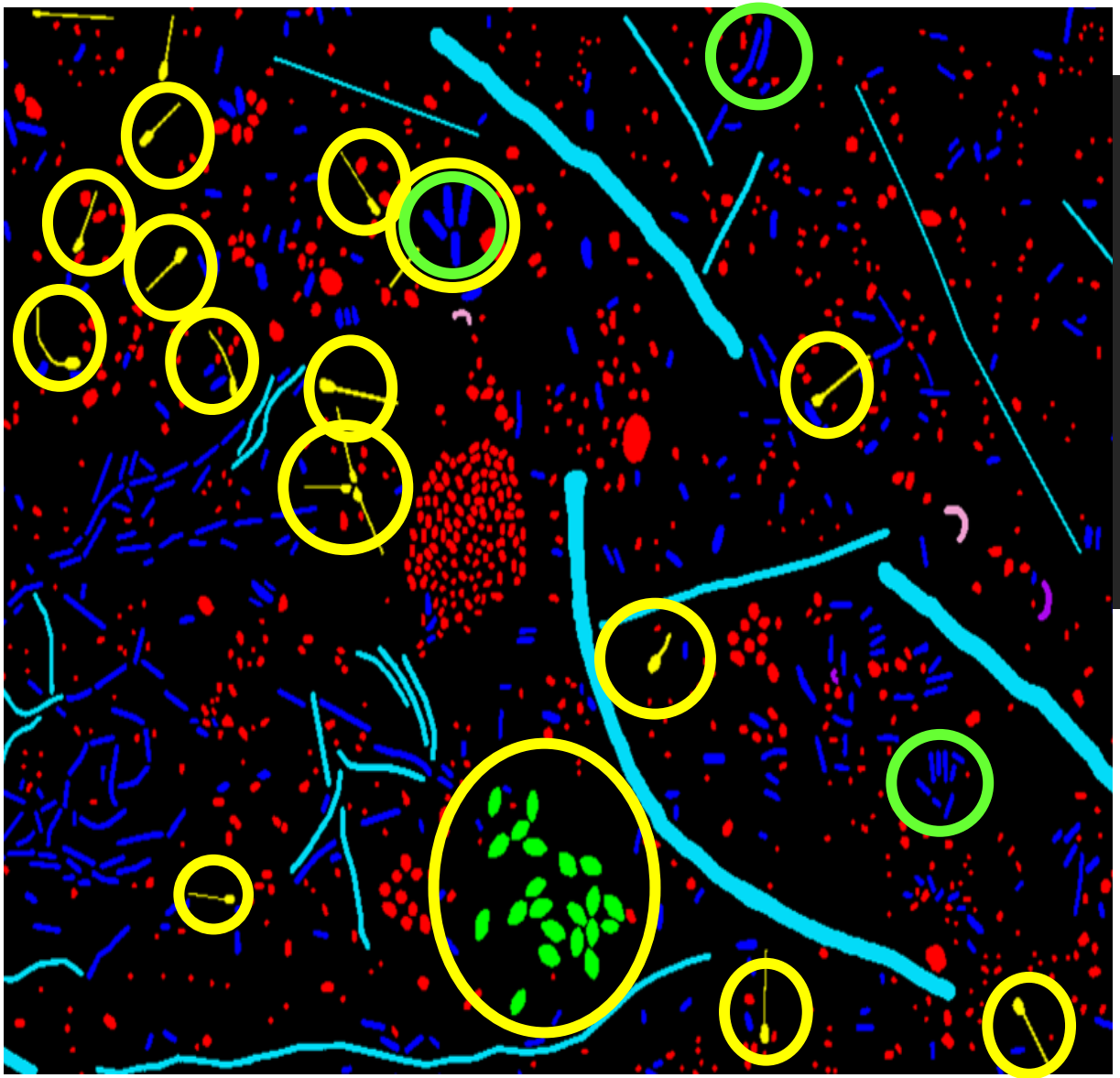
- **Culturability barriers: exist at both the enrichment and isolation stages. We cannot grow all types.**
- **Bias toward fast growers: (‘weeds’) – are these representative of the types in the initial sample?**
- **Too many variables: Many inoculum, media, and incubation conditions…which are most important?**

Dandelion + Eggplant

Sharing same soil patch

Select for fast growth over many generations

Enrichment & isolation: limitations



Grows in enrichment

Grows on agar

Choice of enrichment or isolation or both influences type of microbes you get

Can also choose ‘none of the above’ = metagenomics

http://www.mdpi.com/1424-2818/5/3/426

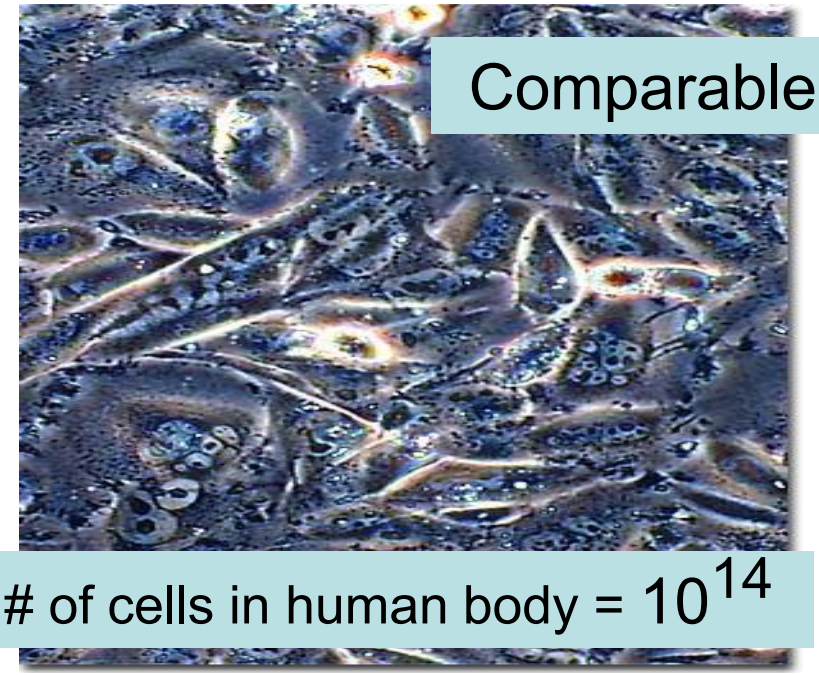
The Metagenome

→ the complete gene content of an environmental sample

- **Calculations for Bacteria alone:**

~ 10^3 genes per genome **X** **~ 10^9 cells per gram of soil** → **~10^{12} genes per gram of soil**

Comparable large numbers



# of cells in human body = 10^{14}

micro.magnet.fsu.edu

# of bits on a hard disk: 10^{12}

flickrhivemind.net/Tags/harddrive,hdd/Interesting

The Metagenome

- **How can we effectively sample this massive diversity?**

  → **targeted approach: sampling / enrichment**

  → **large-sized inserts of DNA**

  → **large numbers of clones**

  → **high-throughput screening assays**

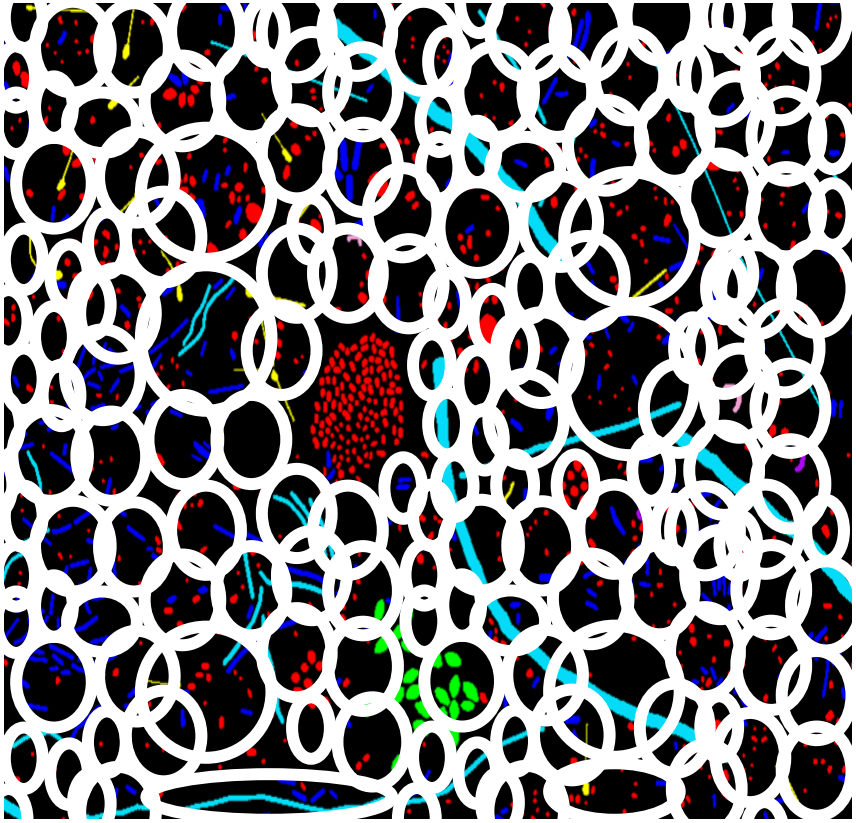
Robot for manipulating 384-well plates

…or get an Honours student with a p-200 pipette and a very steady hand…☺

Metagenomics vs. isolation for bioprospecting

Metagenomics advantages:

- Gene sources not limited to culturable organisms
- Greater NUMBER and DIVERSITY of genes available



Metagenomics limitations:

- Hard to find multi-gene phenotypes (impossible if in different genome regions)
- Any functional screen depends on success of heterologous expression in *E.coli* or other host

Clone libraries

What are the reasons for making a clone library?

- **Localises gene of interest to a smaller piece of DNA**
  → enables further analysis e.g. sequencing
  → info on genomic context (other genes nearby)

- **Facilitates expression of cloned gene(s)**
  → Expression is crucial for function-based screening
  → promoter & RBS could be in vector, or in insert DNA

Overview of clone library construction

Sample (microbe, enrichment, soil…)

DNA extraction

Genomic or Metagenomic DNA

Plasmid or other vector

Restriction digestion

sticky-end genomic fragments

sticky-end vector

Ligation

Ligation mixture

Transformation into host

Individual clones (e.g. colonies on agar)

Screening

Cloned gene of interest

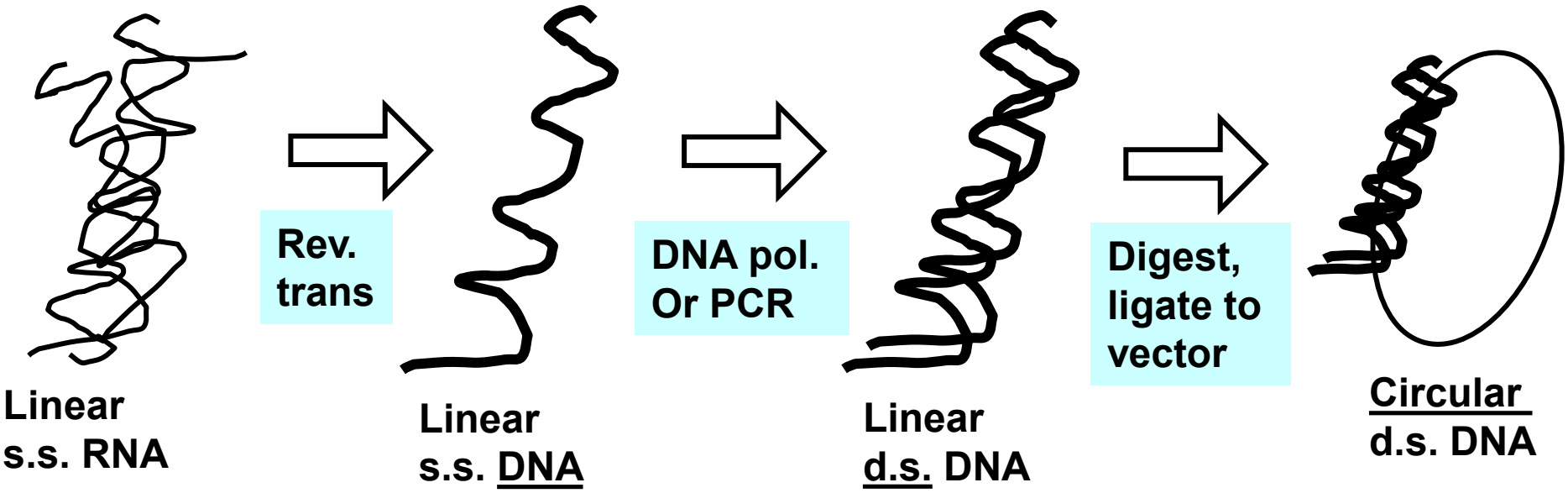
expression, purification

Recombinant protein

Can you find useful PCR shortcuts?

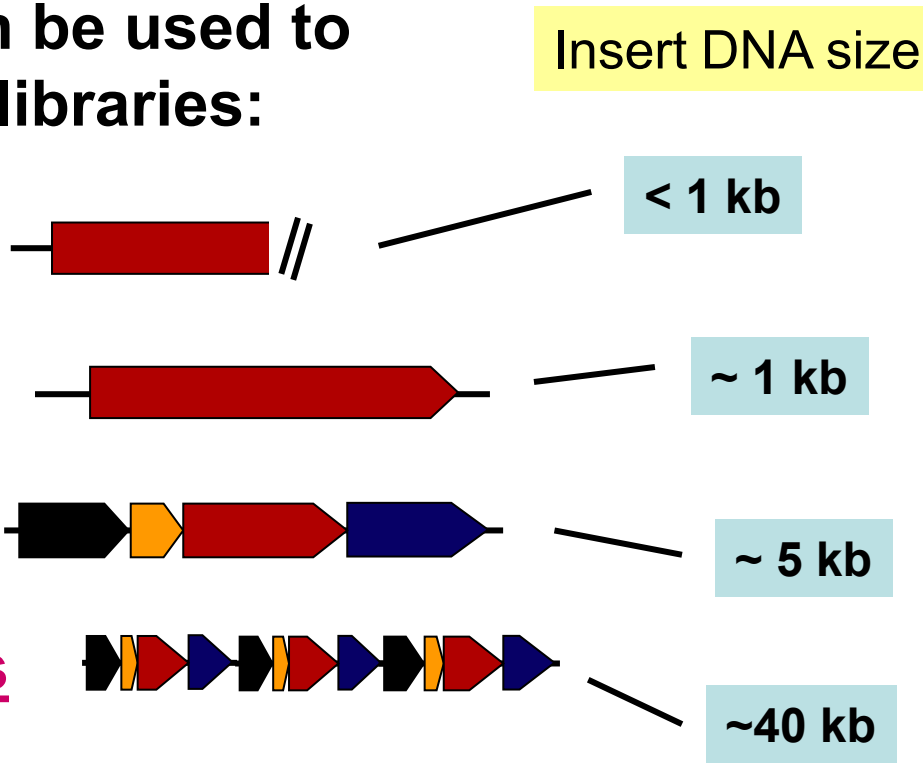
Clone library construction – template?

- Nucleic acids can come from one microbe or a mixture → gives genomic or metagenomic library, respectively
- Clone libraries can be made from DNA or RNA; with RNA, need to reverse-transcribe to cDNA first
- Different extraction, cloning, screening methods are needed, depending on source & type of nucleic acids



Clone library construction – insert DNA size?

- **The size of insert DNA can be used to categorise types of clone libraries:**



  - **Gene fragments**
  - **Whole single genes**
  - **Gene clusters**
  - **Large genomic regions**

- **Different vectors, DNA extraction, cloning, and screening methods are needed for different size inserts**

Clone library construction – insert DNA size?

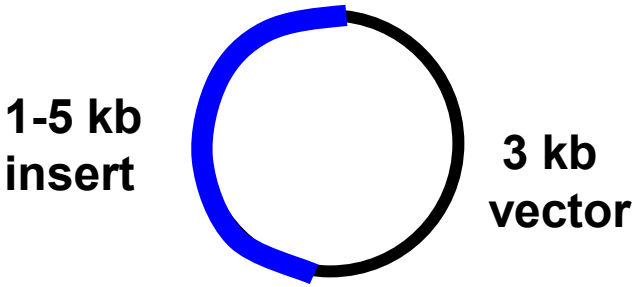
How does insert DNA size affect the cloning method and the applications ?

	Obtain by PCR amplific. *?	Obtain by direct cloning ?	Builds seq. database ?	Useful for exp. evolution	Gives active enzyme ?
Gene Fragment	+	+	+	+	--
Whole gene	--	+	+	+	+/-
Gene cluster	--	+	+	+	+

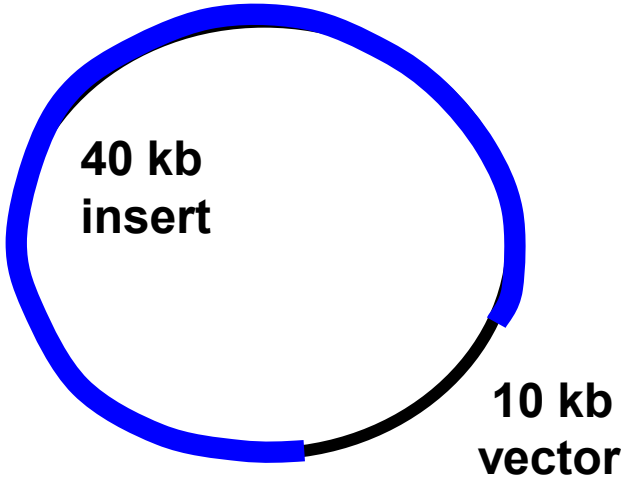
***PCR here is limited by need to target conserved sequences – these not always at ends of genes

Choosing the right vector:

- large vs. small inserts



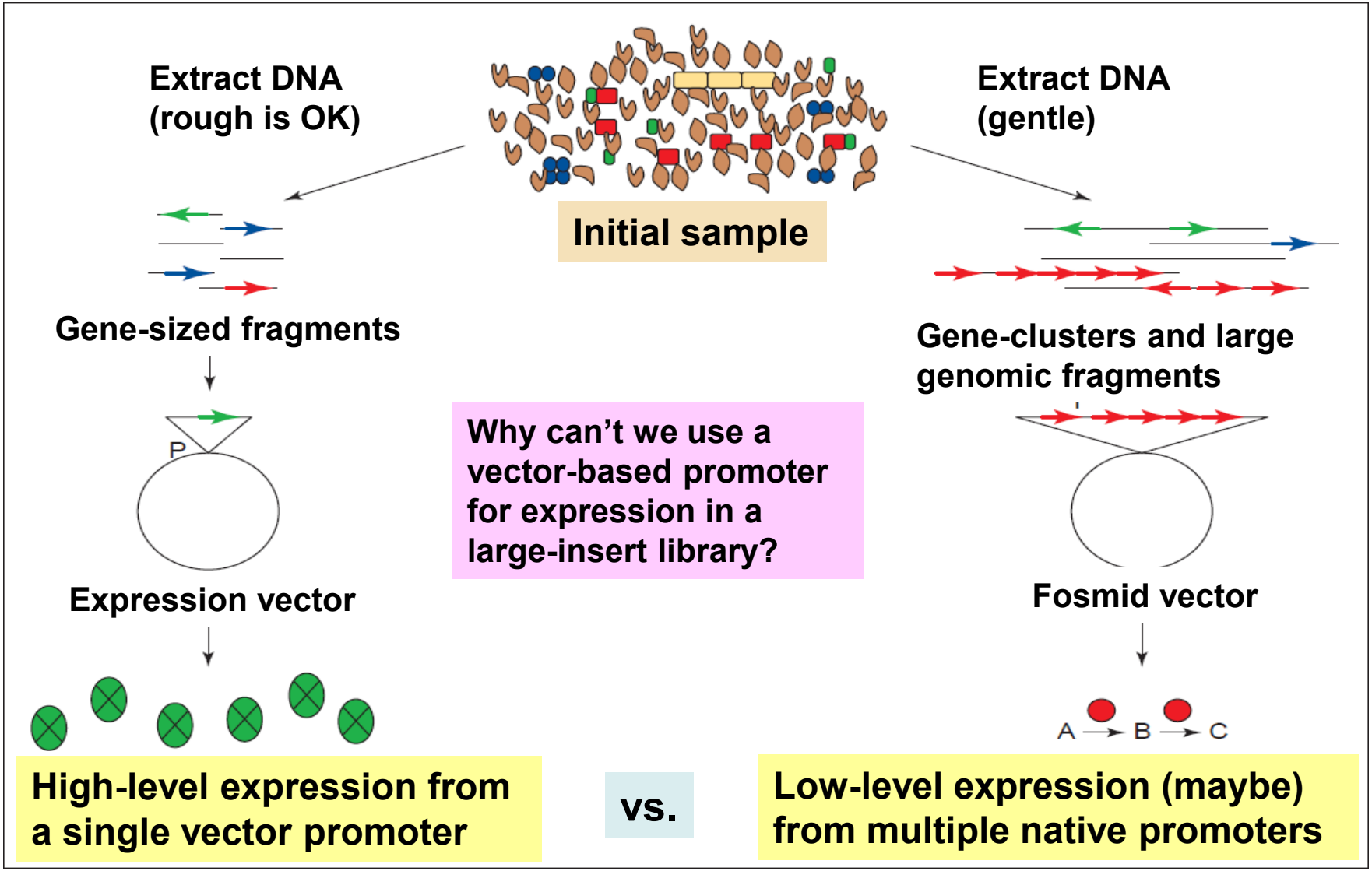
vs.



	pET / pUC / pGEM	Fosmid / BAC
Library construction	easy	hard
Insert sequencing	easy	hard
Chance of intact genes	low	very high
Chance of gene clusters	very low	high

Clone library construction

→ insert size determines gene expression strategy

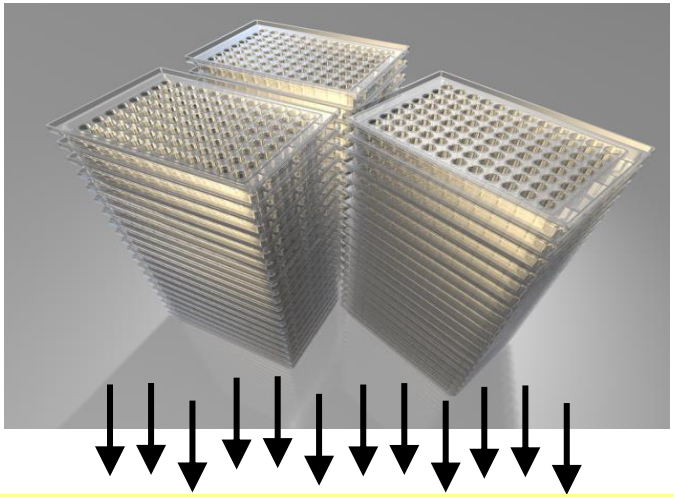


Rondon *et al.* (1999) The Earth's bounty: assessing and accessing soil microbial diversity. *Trends in Biotechnology* **17:**403-409.

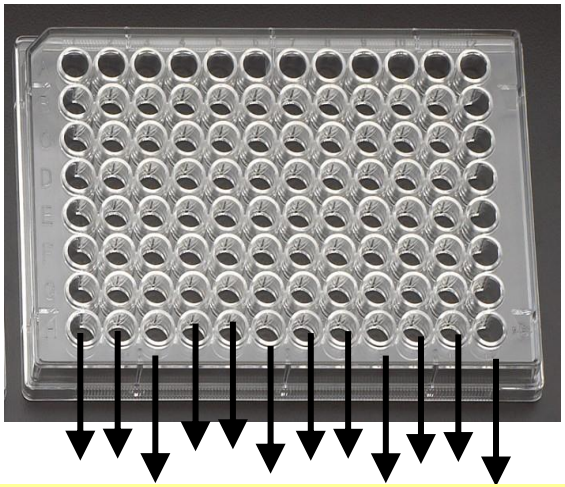
Sequence-based screens: PCR

- **PCR screening is quick and easy to do; most microbiol. labs have the equipment needed.**
- **PCR can be done direct from E.coli colonies on agar plates, or in mini-broths (e.g. 96-well microtitre plates)**
- **Can pool the clones before PCR → enables screening of larger numbers → then backtrack to indiv. clone.**

http://thedude.com/2012/



positive plates



globebio.com

positive columns

Pool entire plates, PCR-screen

Pool columns (8 wells); PCR-screen

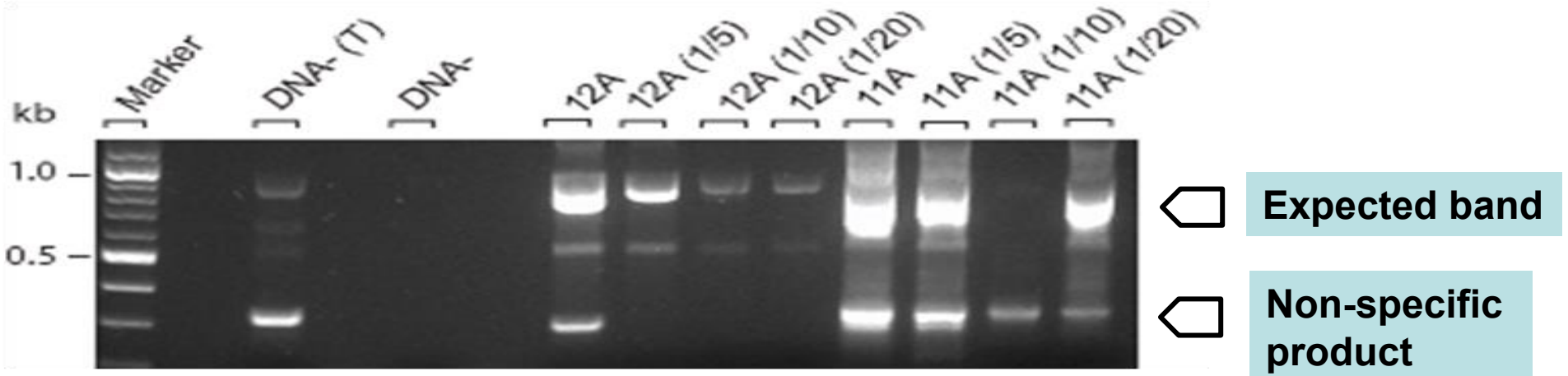
Sequence-based screens: PCR

- **PCR will only yield positive results with clones that have approx >90% identity to BOTH primer sequences**

  → PCR screen will miss much genetic diversity unless degenerate primers are used

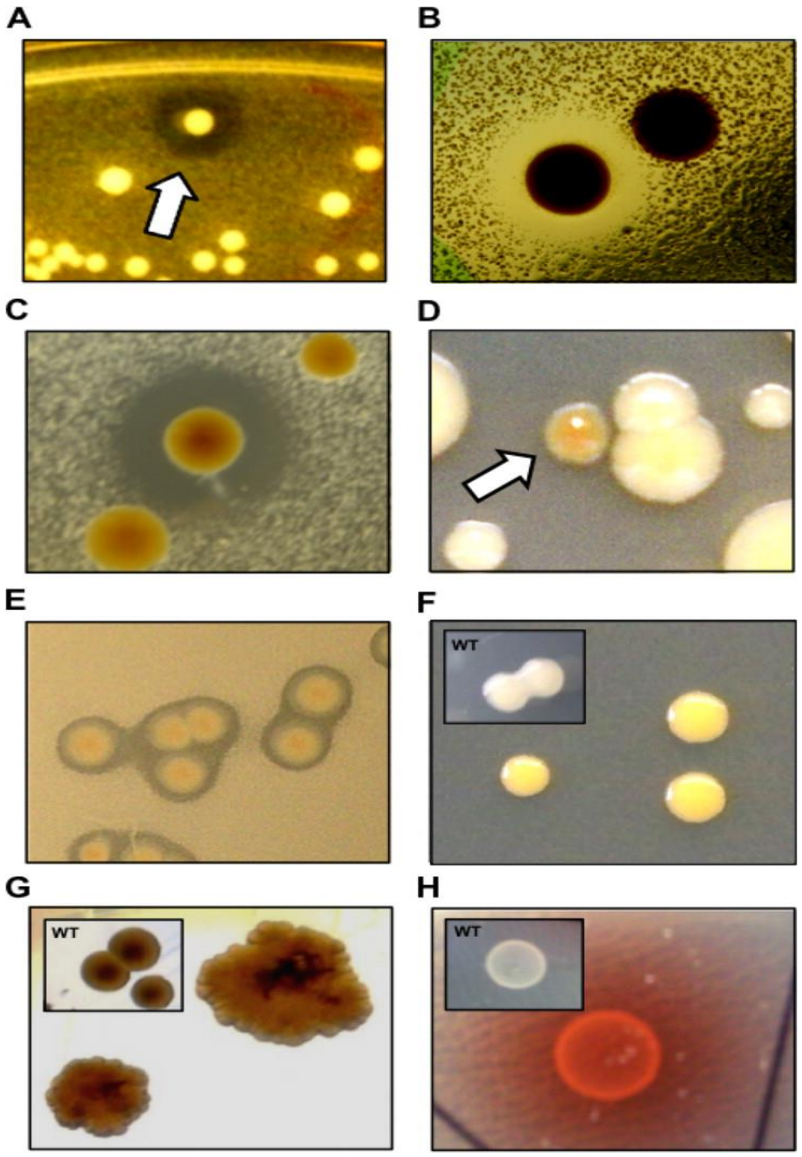
  → but degenerate primers are more prone to problems, e.g. false positives and negatives

- **In all cases, good primer design is critical !**



Simon Dingsdag, Hons thesis

Functional screening of libraries



Craig et al. (2010) Appl. Environ. Microbiol. 76(5):1633-1641

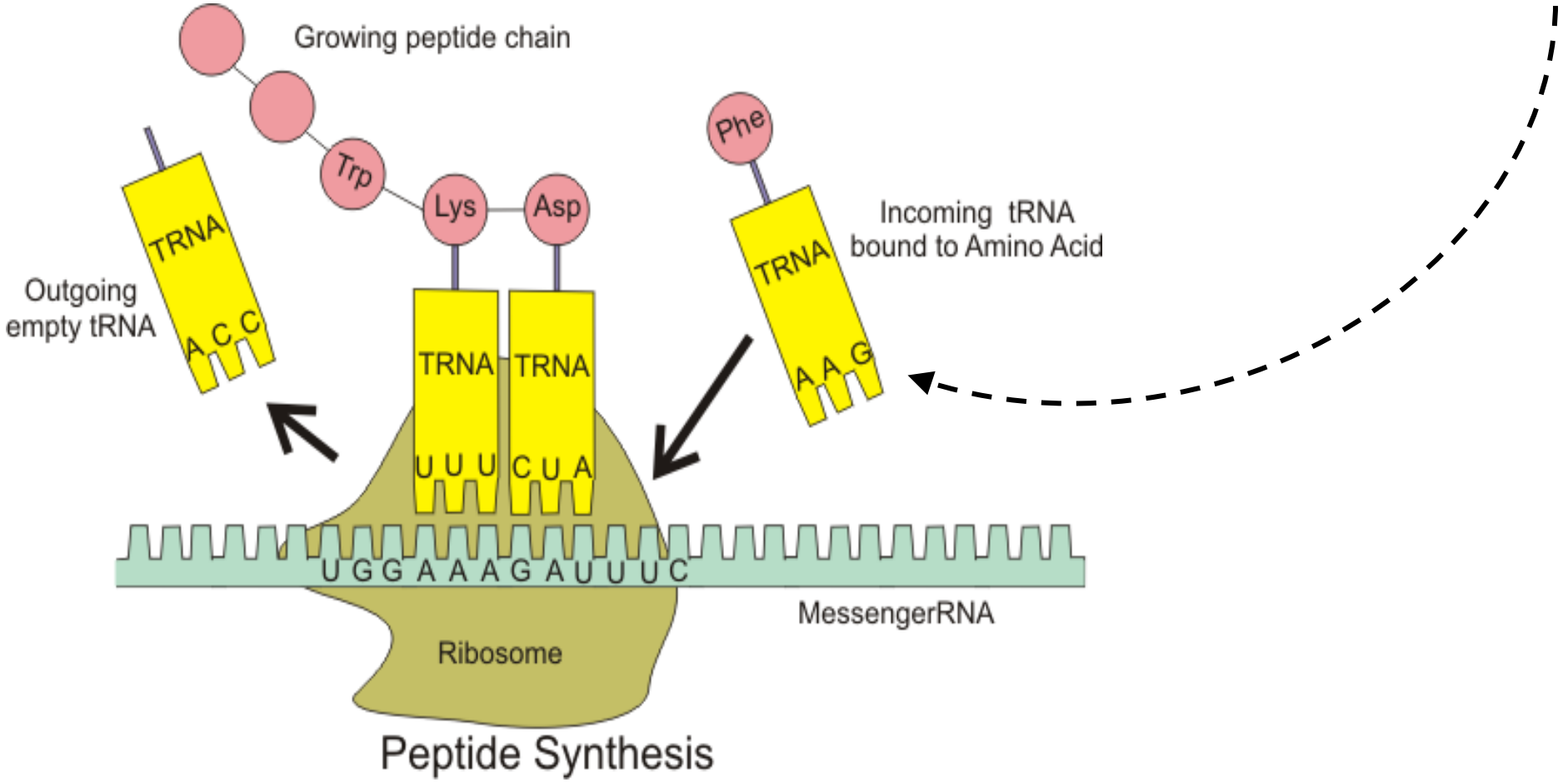
The gene(s) of interest must give phenotypic differences that are readily visible e.g :

- different colour colony
- different shaped colony
- clearing zone around colony

Observing these effects may require special medium additions (chromogenic substrates)

Functional screening of libraries – issues…

- **Is codon usage problematic?** → **What does this really mean?**
- **How will promoter and RBS be provided?** **Vector / native**
- **Is the foreign protein toxic to *E.coli*?**



Practice questions

Describe three different ways in which you could optimise a metagenomic cloning and screening experiment to increase your chances of finding genes coding for amylase enzyme in a metagenomic library.

Gene fragments recovered from the environment by PCR or direct cloning can be useful for biotechnology. Explain how gene fragments can be used, and the limitations of this approach.