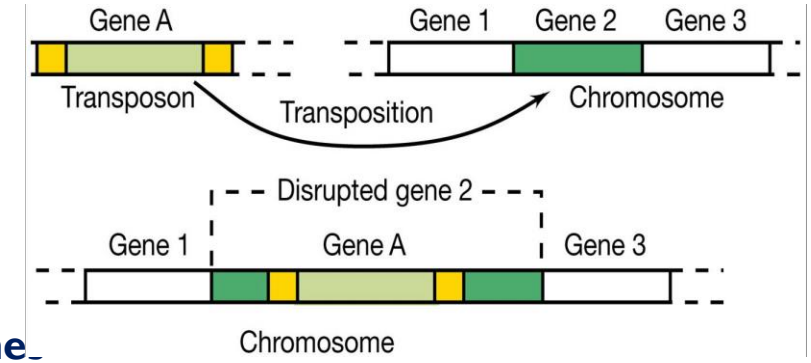
Chapter 5 Molecular approaches to study virulence factors

a.A 2020-21

Approaches used to study new virulence factors

Traditional approaches
Biochemical: purification of virulence factors (diphtheria and cholera toxins)
Molecular genetics: transposon mutagenesis, reporter gene fusion

Finding genes that are expressed *in vivo*
Signature tagged mutagenesis (STM)
In vivo Expression technology (IVET)



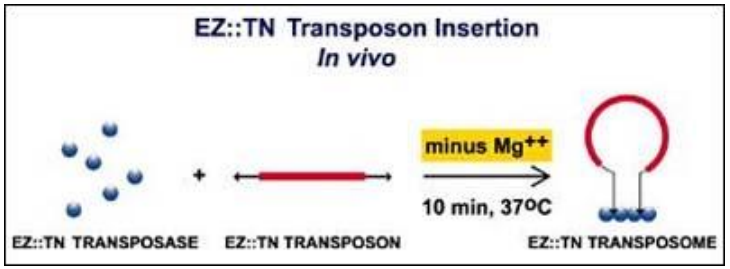
Genomic method for identifying virulence gene
Genomic Subtractive Hybridization (GSH)
Massively parallel methods of DNA sequencing (Next-generation sequencing-NGS)
In Vivo Induced Antigen Technology (IVIAT)

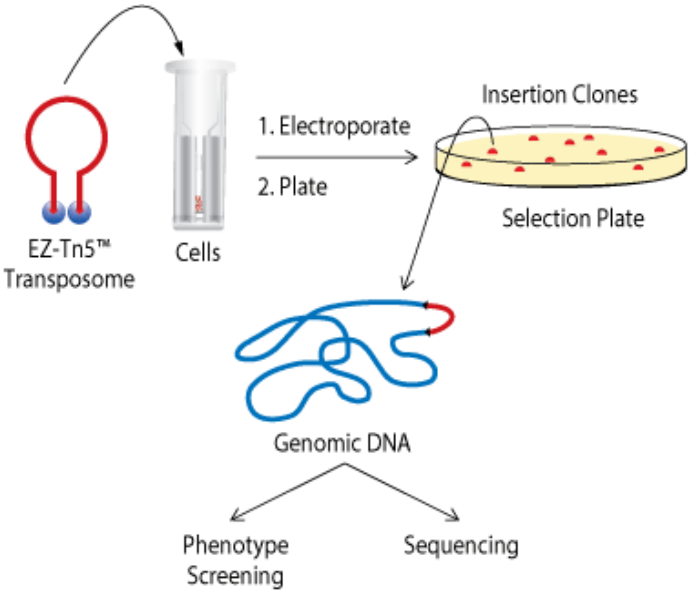
Measuring Infectivity and Virulence
Animal models of infection
Cell and organ cultures.

Transposon mutagenesis

TM is an approach to generate strains with attenuated virulence compared to the wt parent strain and to identify genes responsible of the attenuated virulence.

This occurs using 1) a method to produce library of mutant e.g. **random transposon insertion (**or chemical mutagenesis, UV irradiation,) and 2) a biological assay to screen for those mutants that still grow in the medium but display attenuated virulence.





Method: transposon of choice (with marker) completed of transposase (see figure) is electroporated into competent bacteria of choice.

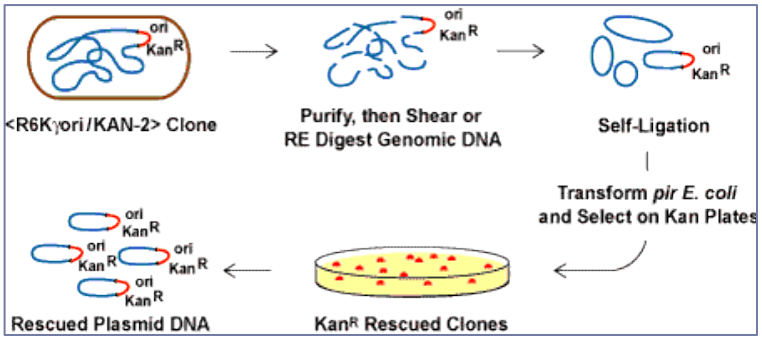
Transformed bacteria are selected on the appropriate medium containing the selective marker (e.g. kanamycin).

Surviving colonies contain each a transposon randomly inserted into their genomic DNA (library).

Transposon mutagenesis: identification of the virulence gene

n colonies are screened for a **certain phenotype** with attenuated virulence (e.g. lost capability to invade tissue culture cells, to survive in macrophages…)

The gene interrupted the by transposon insertion and responsible for the mutant phenotype is identified (see the figure).



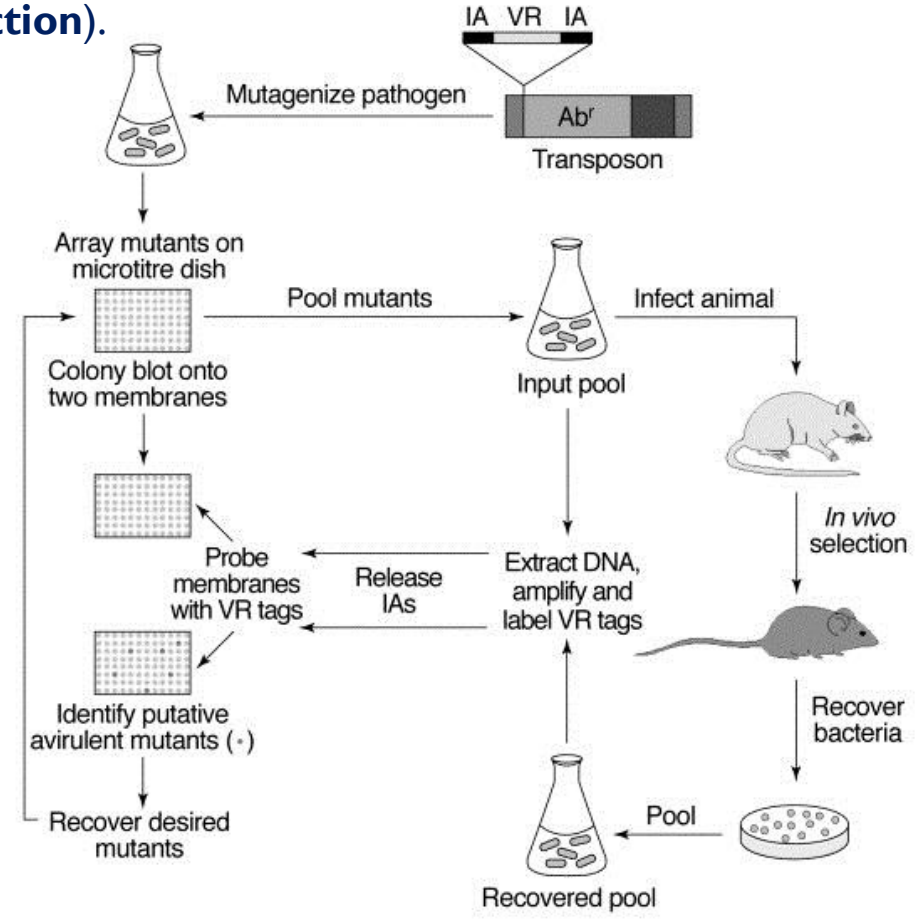
The wt gene is introduced into the mutant strain to verify the restoration of the wt phenotype (complementation test) and to confirm the role of the gene in the virulence.

Limitations: mutants have to be tested individually for altered virulence.
In vitro screening only.
Transposon insertions have often a polar effect.
genes have not to be essential.

Finding Genes that are expressed *in vivo*: STM

Signature-tagged mutagenesis (STM): approach which combines *in vitro* transposon mutagenesis with *in vivo* selection using an animal model of infection.
It allows to identify genes **essential for the survival in the host** by screening for mutants that lost the ability to grow in the host (**negative selection**).

- STM uses a mixture of transposon variants to generate a library of mutants each with a different variant inserted.
- The library is inserted into the target bacteria and selection of the transformants performed by the selective marker (Km^r).
- Individual colonies (representing each a different random mutation) are distributed on masterplate (array mutants).
- DNA is extracted by the masterplate and transferred on two different replicate membranes for probing the samples.
- Mutants are pooled, grown and splitted in 2 parts: the input pool of mutants (control), and a part inoculated an animal to perform the *in vivo* selection (recovered pool).
- After a time (depending from the type of infection) an organ or blood is recovered and bacteria plated in vitro.



Miller
Trends in Microbiology, 1999 Vol. 7, Issue 10, p388

Finding Genes that are expressed *in vivo*: STM (II)

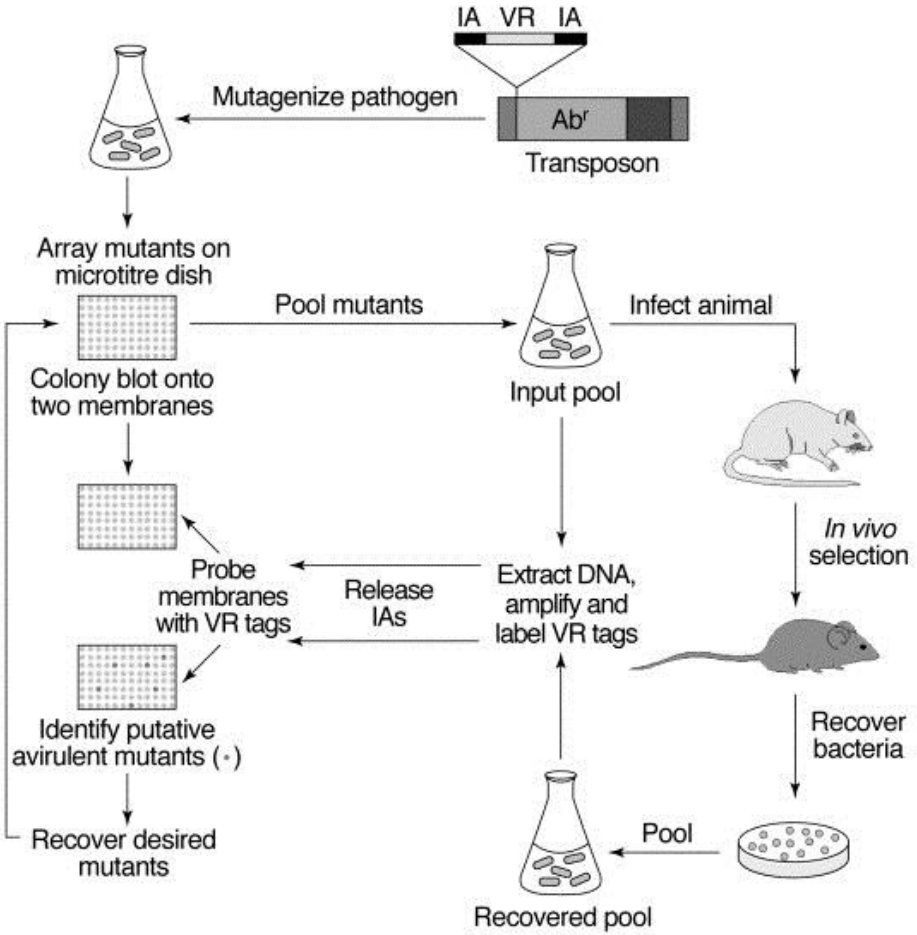
•DNA is extracted from the both pools of bacteria and amplified using the DNA primers (labeled nucleotides) designed on the invariant oligomer tag sequence.

•The amplified DNA is used to probe the membrane containing replicates of DNA from the original collections.

•Those mutants from the output that do not hybridize with the mixture of probe tags represent **mutants lost during the infection.**

Mutants identified as "lost" in the host may arise from genes required for growth in the host:

1) needed to acquire, biosynthesize or metabolize a nutrient;
2) virulence genes needed for colonization, invasion, or dissemination in the host.

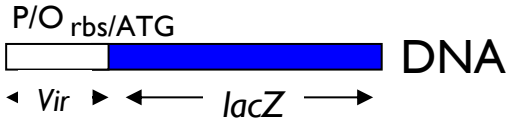


Miller
Trends in Microbiology, 1999 Vol. 7, Issue 10, p388

In Vivo Expression Technology (IVET)

Virulence genes are usually subjected **to differential regulation** under selective conditions (e.g. ON when in the host).
IVET: a method for identifying genes that are expressed when bacteria are infecting an animal host. It is based on the identification of those **promoters** (**promoter trap technology**) that are **turned on** when the bacteria infect a host organism and turn off when bacteria are grown on laboratory medium.

Experimentally characterization of the regulatory expression properties of (virulence) genes can be performed using **transcriptional fusion:**



Transcriptional fusions: hybrid gene with promoter and regulatory regions of the gene of interest + structural gene (*reporter gene*) coding for a reporter enzyme that can be easily detected using several techniques (colorimetric, spectrophotometric, fluorescence or luminescence techniques).

reporter genes examples: β-galactosidase (*lacZ*), β-glucuronidase (*iudA*), GFP (*gfp*), Chloramphenicol acetyltransferase (*cat*) alkaline phosphatase (*phoA*) firefly luciferase *luc*), luciferase (*lux*). A gene that encodes a selective marker that is required for survival

IVET: in vivo Expression Technology

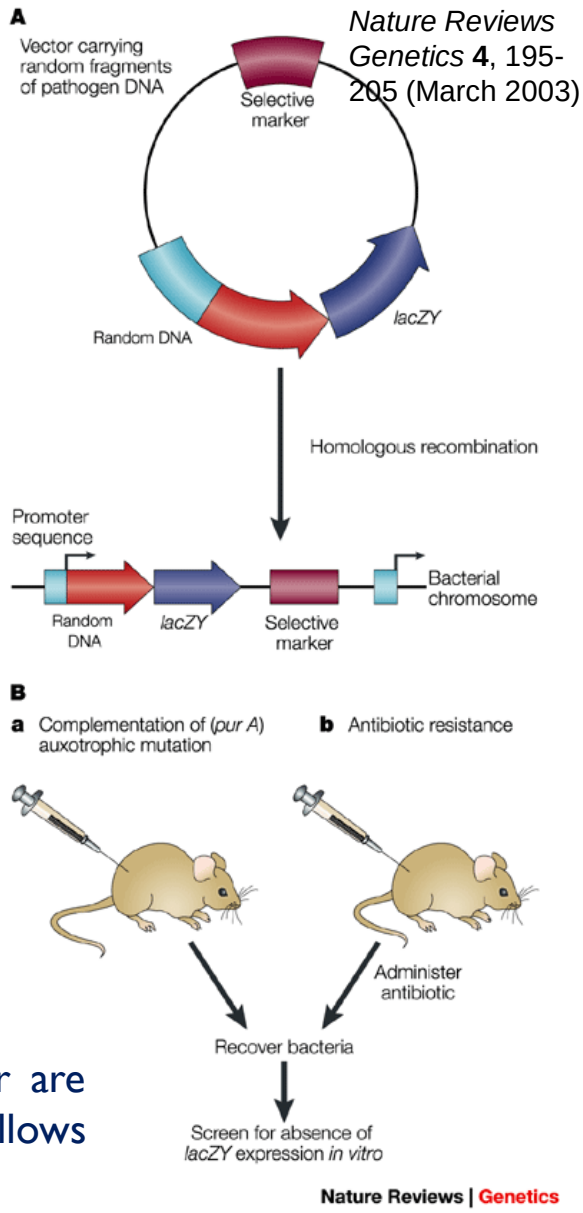
IVET promoter trap strategy is based on the **complementation of auxotrophic mutation** as gene reporter.

It uses vectors contain a random fragment of the chromosome of the pathogen (red) and a promoter-less gene reporter that encodes a **selective marker** that is required for survival (dark red).

Random integration of the IVET vector into the pathogen chromosome of a auxothophic mutant (e.g. ΔpurA= **a purine auxotrophs unable to grow in the mice**) is performed by insertion–duplication mutagenesis to create a pool of recombinant pathogens. Pooled clones are then inoculated into the mouse

Only those bacteria that contain the selective marker fused to **a gene that is transcriptionally active** in the host complementing of auxotrophic mutation are able to survive.

After a suitable infection period, bacteria that express the marker are rescued from infected organs. The inclusion of *lac*ZY gene (blue) allows post-selection screening for promoters that are only active *in vivo*.

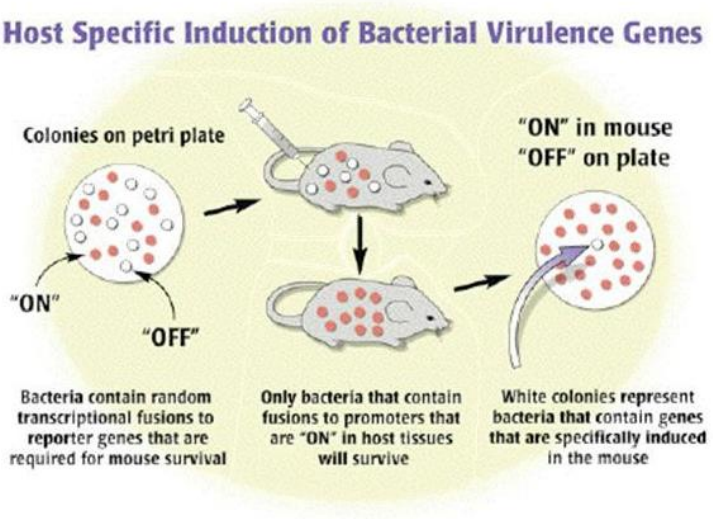


Nature Reviews Genetics **4**, 195-205 (March 2003)

IVET to identify virulence genes

STM and IVET have been successfully used for the identification and characterization of several genes of different pathogens.

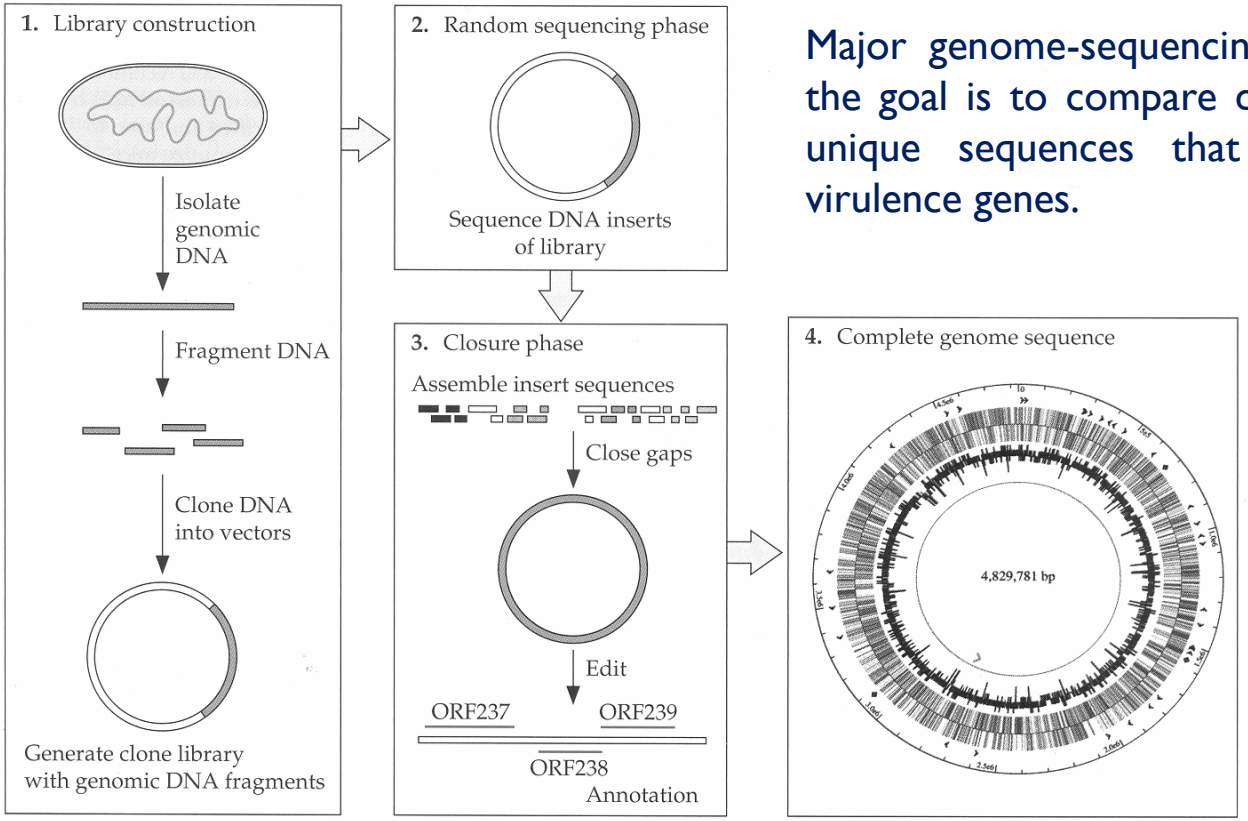
•IVET advantages/limits
•It is a selection that identify only promoters not single genes. More work is needed to examine putative virulence downstream genes
•A high transcription level *in vivo* is mandatory
•Genes products that are activated in the host by a posttranscriptional mechanism may be missed.



Auxotrophic strains of different pathogens are not common. The expression of antibiotic resistance can be used as alternative to the complementation of auxotrophic mutation.

STM advantages/limits:
•It is applicable to a wide range of bacteria. Selection is fast (when the transposon library is made).
•Transposon are not available for all bacteria and insertions are not completely random.

Step in whole-genome shotgun sequencing



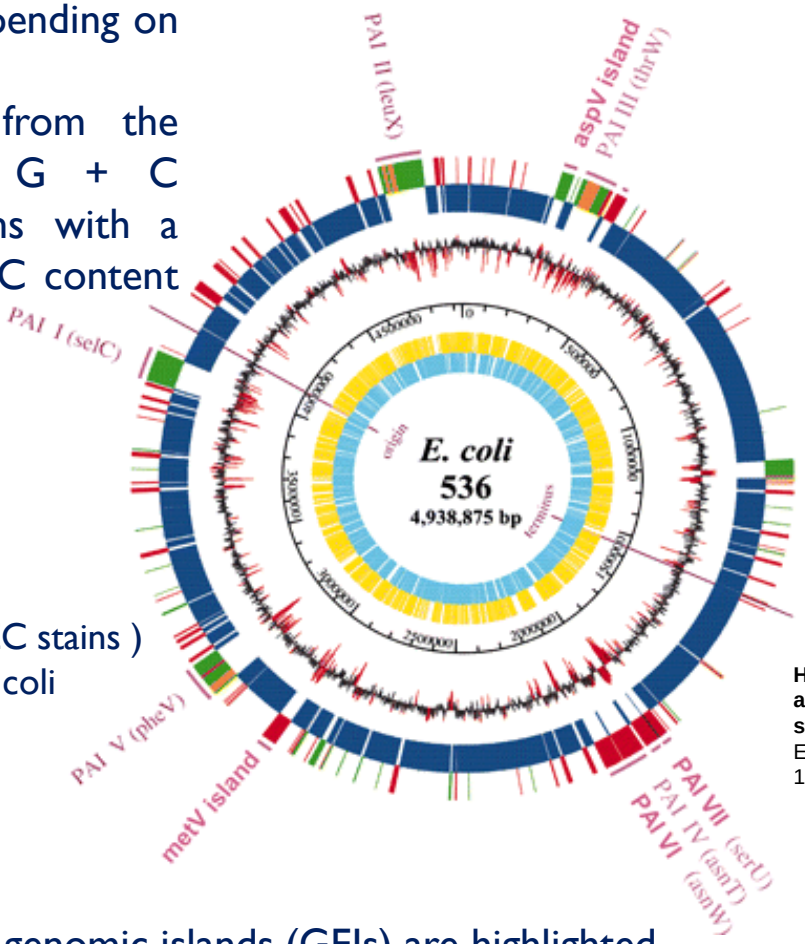
Major genome-sequencing projects are under way: the goal is to compare different strains and to find unique sequences that might be strain-specific virulence genes.

Steps in whole-genome shotgun sequencing

In shotgun sequencing, DNA is broken up randomly into numerous small segments, which are sequenced to obtain *reads*. Multiple overlapping reads for the target DNA are obtained by performing several rounds of this fragmentation and sequencing. Computer programs then use the overlapping ends of different reads to assemble them into a continuous sequence.

Genetic map of the UPEC strain 536 chromosome

The two inner circles represent all putative genes, depending on ORF orientation.
The fourth circle from the center shows the G + C distribution. Regions with a highly aberrant G + C content are in red.



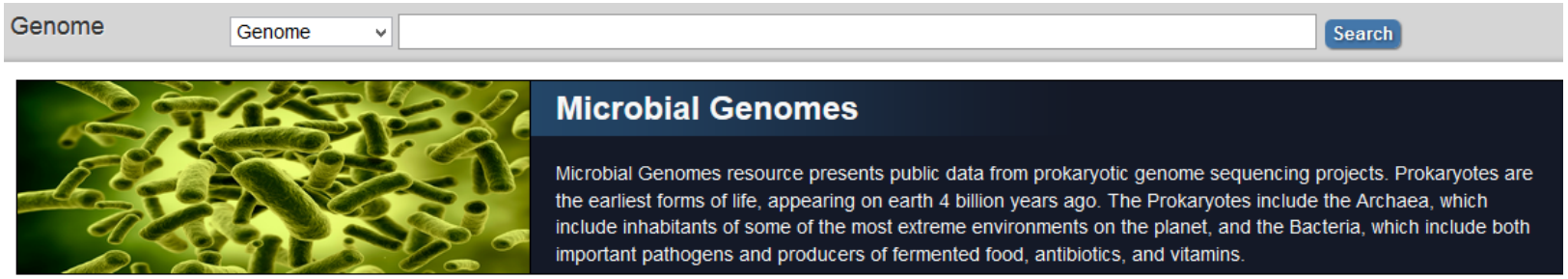
The outermost circles show the result of a three-way genome comparison with the UPEC strain CFT073 and *E. coli* MG1655 (K-12) genomes.
Blue: backbone genes found in all three strains; red, genes present in 536 and CFT073 but absent from MG1655; green, genes found in 536 only.

536 and CFT073 = UPEC stains)
MG1655 = reference E. coli

How to become a uropathogen: Comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains
Elzbieta Brzuszkiewicz, et al. PNAS August 22, 2006 103 (34) 12879-12884;

Pathogenicity and genomic islands (GEIs) are highlighted

Microbial genomes resource

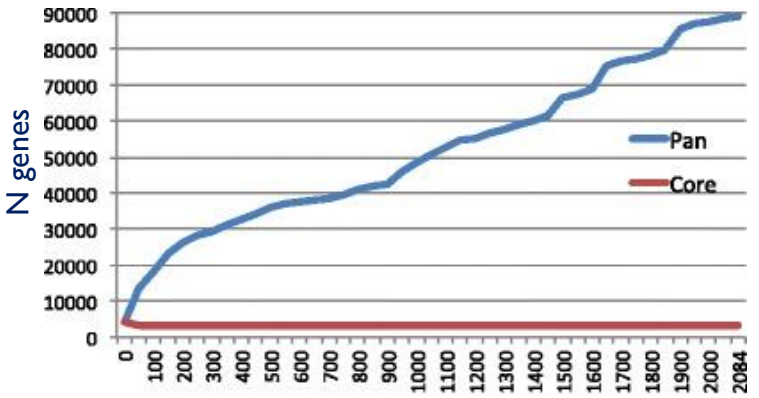


Sequencing status can be monitored at: https://www.ncbi.nlm.nih.gov/genome/microbes/

(10/05/2018) Eukaryotes (5657) Prokaryotes (139120) Viruses (15507) Plasmids (12745) Organelles (11722)
(16/11/2018) Eukaryotes (6817) ; Prokaryotes (170843);Viruses (21184); Plasmids (14309); Organelles (12116)
(20/11/2019) Eukaryotes (9535); Prokaryotes (218539); Viruses (33719); Plasmids (19082); Organelles (15011)

Depending on the species, the variation in gene content and genome size can be quite considerable, with some pan-genomes, like *E. coli*, being very "open".

Core and pan-genome of 2085 *E. coli* genomes. Core gene families defined as those families with at least one member in at least of 95 % of genomes



n. of E. coli genomes

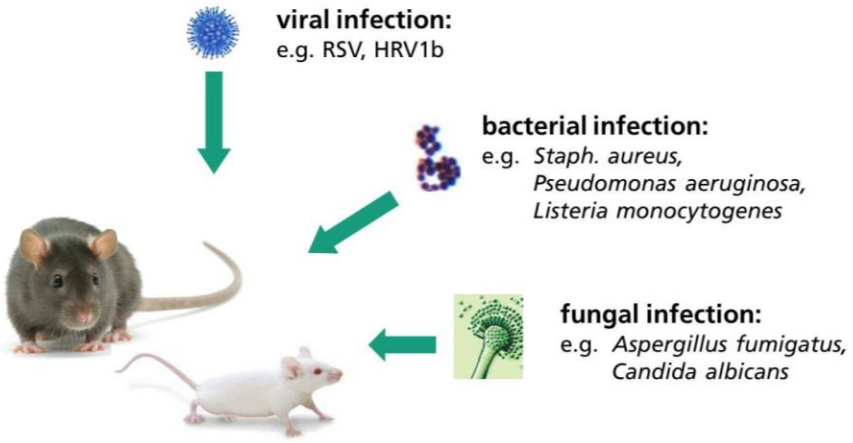
Funct Integr Genomics. 2015; 15(2): 141–161.

Measuring Infectivity and Virulence

Animal models of infections: Gold standard **for infectious diseases research.** Usually rodents are used.

Factors that have to be taken into account:

- Differences with humans: microbiota, in part the immune system,
- Symptoms of disease could be different:
  - *i.e. S. enterica* in mice causes a systemic disease, *S typhi* does not infect mice.
- Use other animals: not closely related but with similar aspect of diseases:
  - Ferrets for gastric ulcers, guinea pigs as model for tuberculosis etc
  - *Caenorhabditis elegans* for *P. aeruginosa* infections etc



Ideal animal model: similar symptoms and body distribution, same route of infection. Similar course of disease

Choice of animal model: other factors.

Many factor to be considered:

-Availability to genetically manipulated animals:

Genetically defective of some immune element

Knockout mice and knock-in mice (human gene introduced into their genome) and germ-free animals.

- how difficult is to maintain animals

Ethical considerations:

Compelling reasons for carrying out these experiments,

Approval of ethical committee is mandatory

There must be a truly strong rationale to use animals.

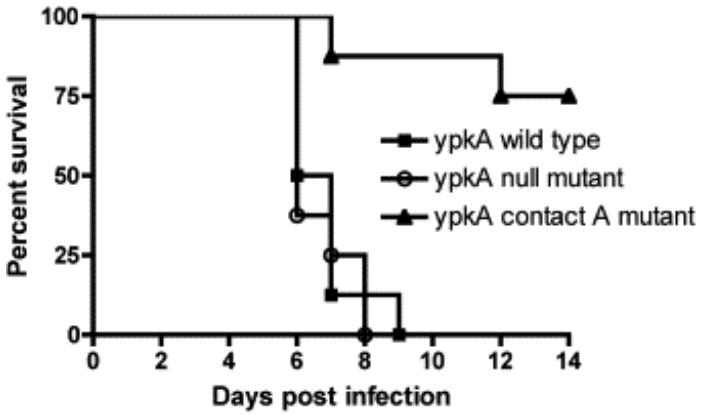
No alternative model can provide information equivalent.

Death of animal should not be used as an end point.

Type of in vivo Infectivity and Virulence measurements

Survival curve analysis: median survival time after infection with wt or mutant is tested for define virulence (on the right).

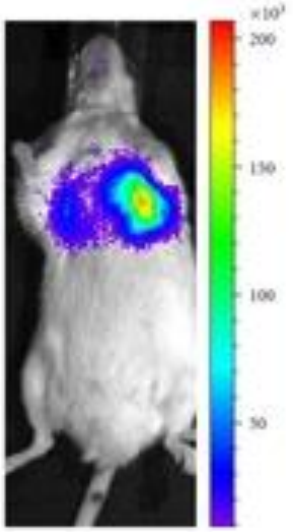
Common measures: LD_{50} ID_{50} : dose responsible to the 50% of animals dead or infected.



G. Prehna et al. Cell, 126, 2006, 869 - 880

Survival-curve analysis (Kaplan–Meier analysis) of mice infected intragastrically with 5×10^9 CFU wt strain and mutants of *Y. pseudotuberculosis*.

Measurements of colony forming units (CFU) in the blood (bacteremia) or in other tissues after mice have been sacrificed.

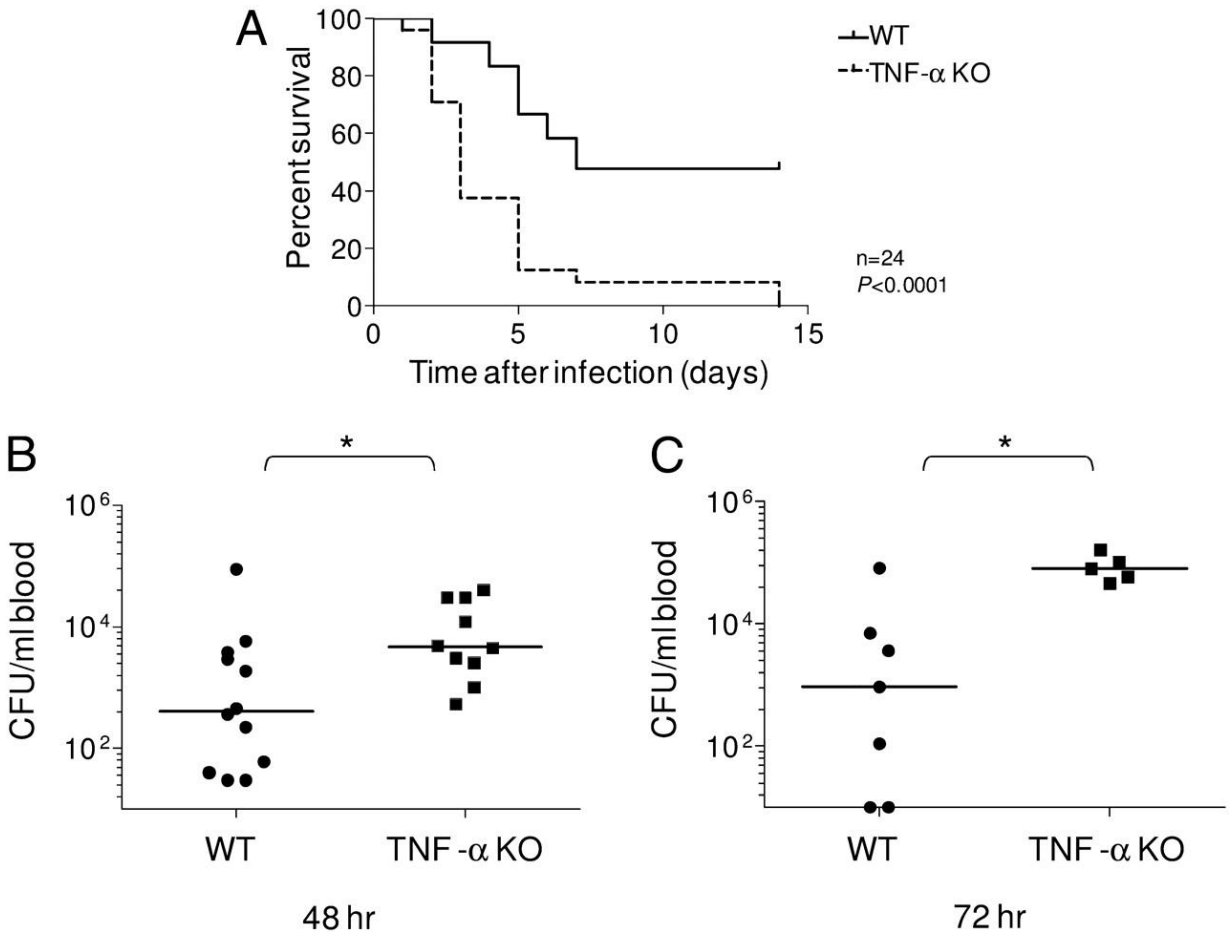


In vivo imaging technology for studies of infectious disease. A bacteria luciferase operon is inserted into bacterial strains and the strain used to infect mice.

Bioluminescence imaging of invasive infection. Light emission is detected from both lung lobes indicating the establishment of bronchopulmonary infection. Bioluminescence was acquired 28 h after infection

M. Brock International Journal of Microbiology Volume 2012 (2012),

Survival-curve analysis and CFU counts of TNF-α deficient mice infected with a bacterial pathogen



WT (C57BL/6) and mutant mice deficient of the cytokine TNF-α were subcutaneously inoculated with 10^8 CFU of a strain of *group A streptococcus*, which was isolated from a patient.

TNF-α KO mice are highly susceptible to the pathogen, and showed a rapidly progressing infection and an enhanced death rate within 2–7 d after challenge.

Inbal Mishalian et al. J Immunol 2011;187:6022-6031

The Journal of Immunology

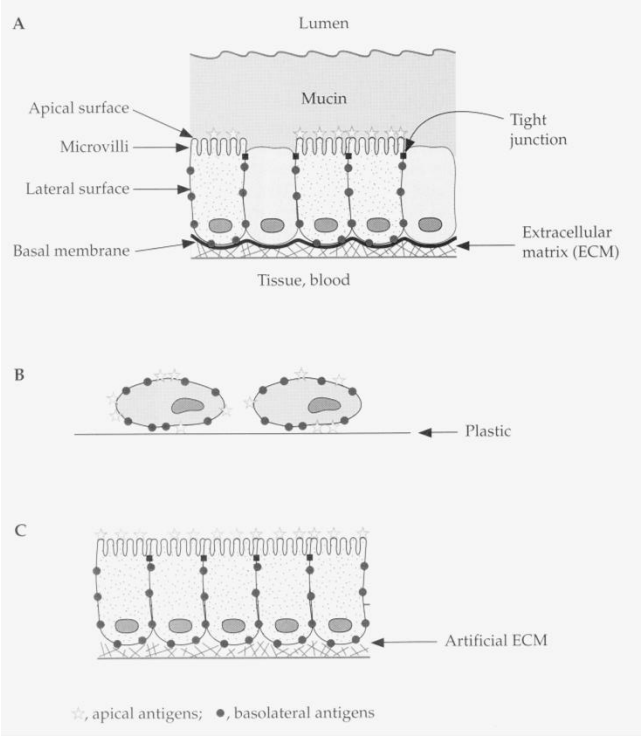
Cell and organ culture models

Primary cultures of mammalian cells and cell lines are used. Provide a more easily controlled system for investigating host–bacterium interactions.

Limitations: cells are no longer in the same environment. They lose many traits of the original tissues: express different markers, often they are not polarized: impossible to reproduce normal mucosa cells (difficult to mimic presence of mucus).
Addition of an extracellular substitute substrate (matrix) for the cells to grow on.

Widely used for studying: adherence properties of bacteria to host cells, attachment of bacteria, cell invasion, intracellular replication, etc.

Organ cultures: more realistic but difficult to obtain and to maintain. Development of artificial organ cultures.



Differences between cells of an actual mucosal membrane and cultured cells
A) In vivo membrane, B) nonconfluent, nonpolarized cultured cells, C) polarized monolayer of tissue culture cells attached to a semipermeable membrane

Slide aggiuntiva (non in programma d’esame)

In Vivo Induced Antigen Technology (IVIAT)

To **identify in vivo-expressed antigens**.
Allow to identify pathogen-associated factors that elicit immune response in vivo: potential candidates for new vaccines and diagnostic tools.

Used **pooled sera from patients** that have been exposed to a certain pathogen to probe an **expression library** obtained from the pathogen and cloned in *E. coli* (of in a phage, phage display).

Proteins recognized by the patient serum are potential **in vivo-expressed antigen**

in vivo induced antigens (IVI) are then (A) inoculated in the animals to confirm their antigenicity; and (B) to test the effectiveness of the protective immune response of animals challenged with the pathogen (vaccination).