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Evolution of antibiotic resistance at sub-MIC

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Two main points

1. Extremely low antibiotic concentrations can drive fast evolution towards high-level resistance
   → clinically important resistance development probably occurs in the environment
   → this type of evolution is probably largely underestimated because of how we do genetics

2. Weak selection generates more problematic resistant mutants

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Studies of resistance selection at very low antibiotic concentrations require:

1. Whole genome sequencing
2. Genetic reconstruction (e.g. Lambda red recombineering)
3. Very sensitive competition experiments

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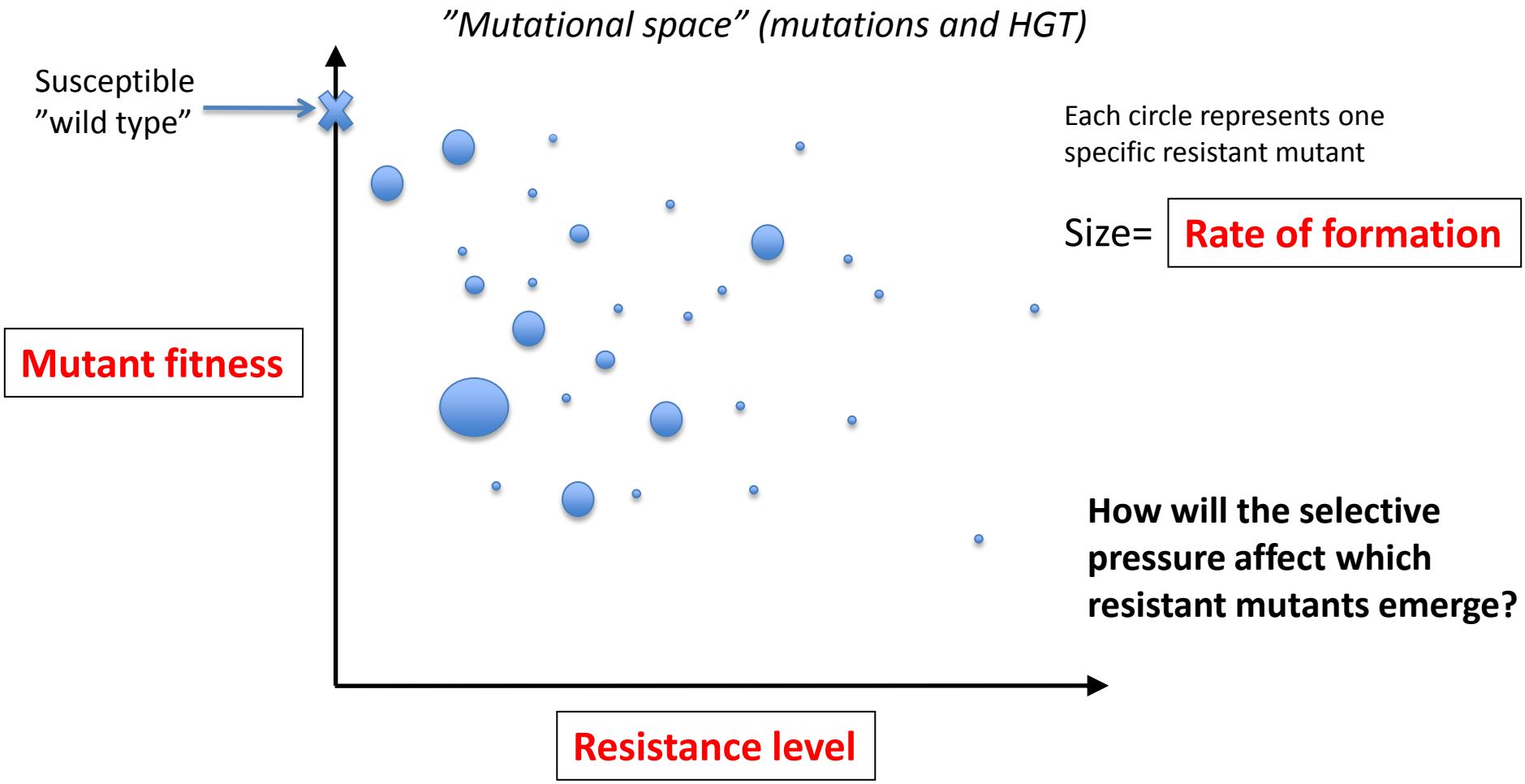
Selection of resistance



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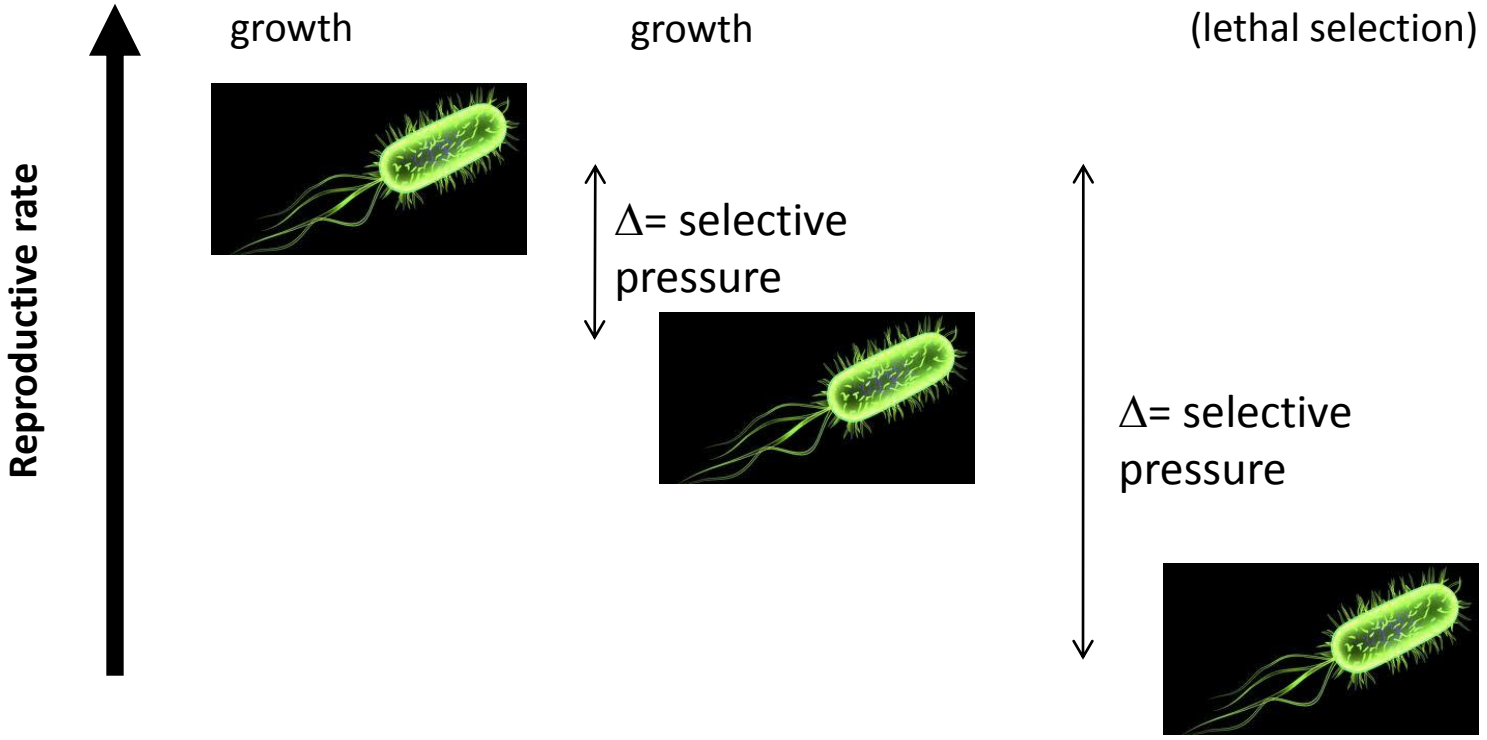
Defining selective pressure

Most of our understanding of resistance evolution comes from using lethal selections



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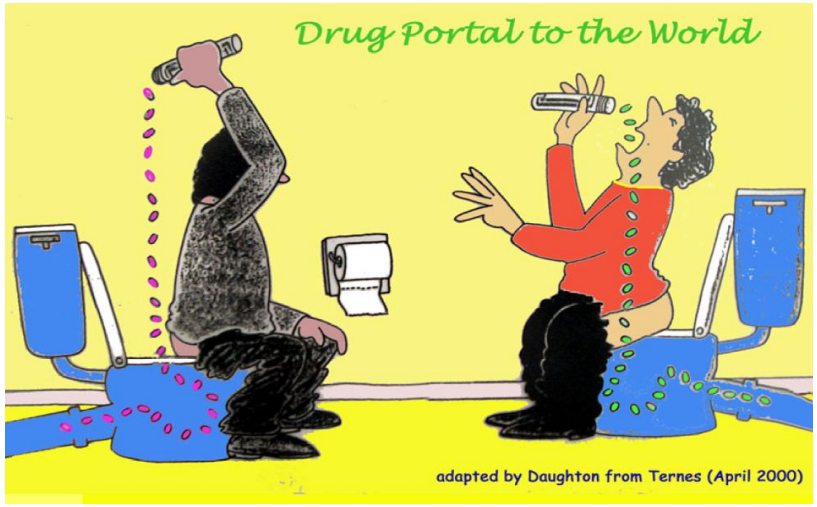
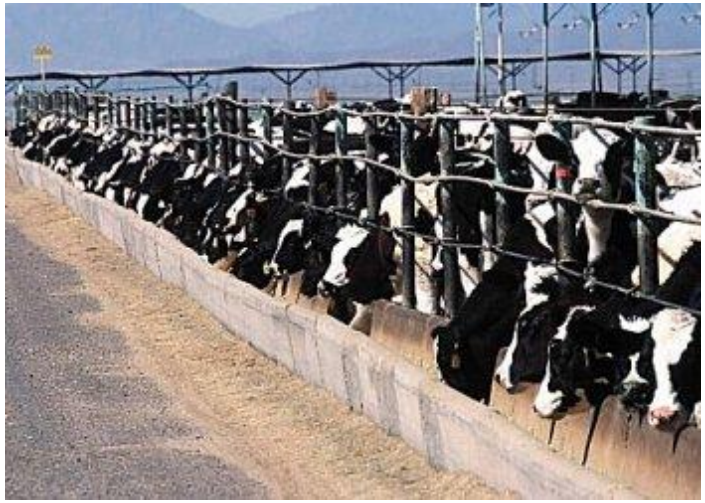
Drugs for human treatment and veterinary use are released into the environment

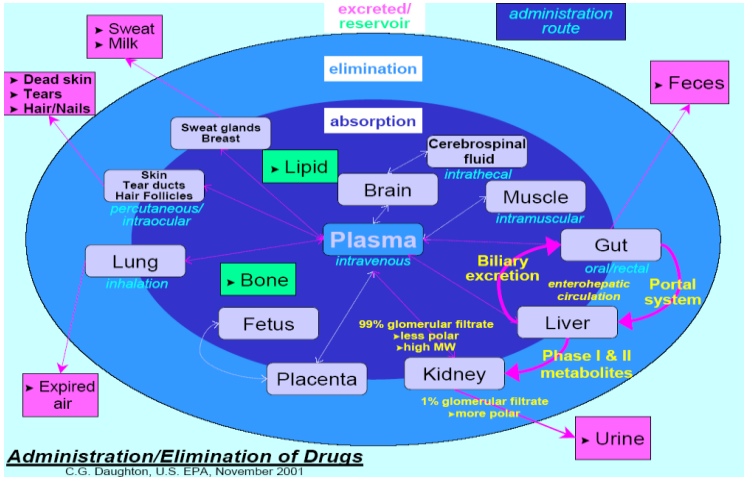
Of the >500,000 tons of antibiotics used per year globally → ≈50% is released in active form into the environment, mainly via human and animal urine





Antibiotics are mainly excreted in urine

	% of dose excreted from humans in active form
Fluoroquinolones	40%
Aminoglycosides	80-90%
Tetracycline	40%
Macrolides	20-30%
B-lactams	50%-90%
Trimetoprim	50%



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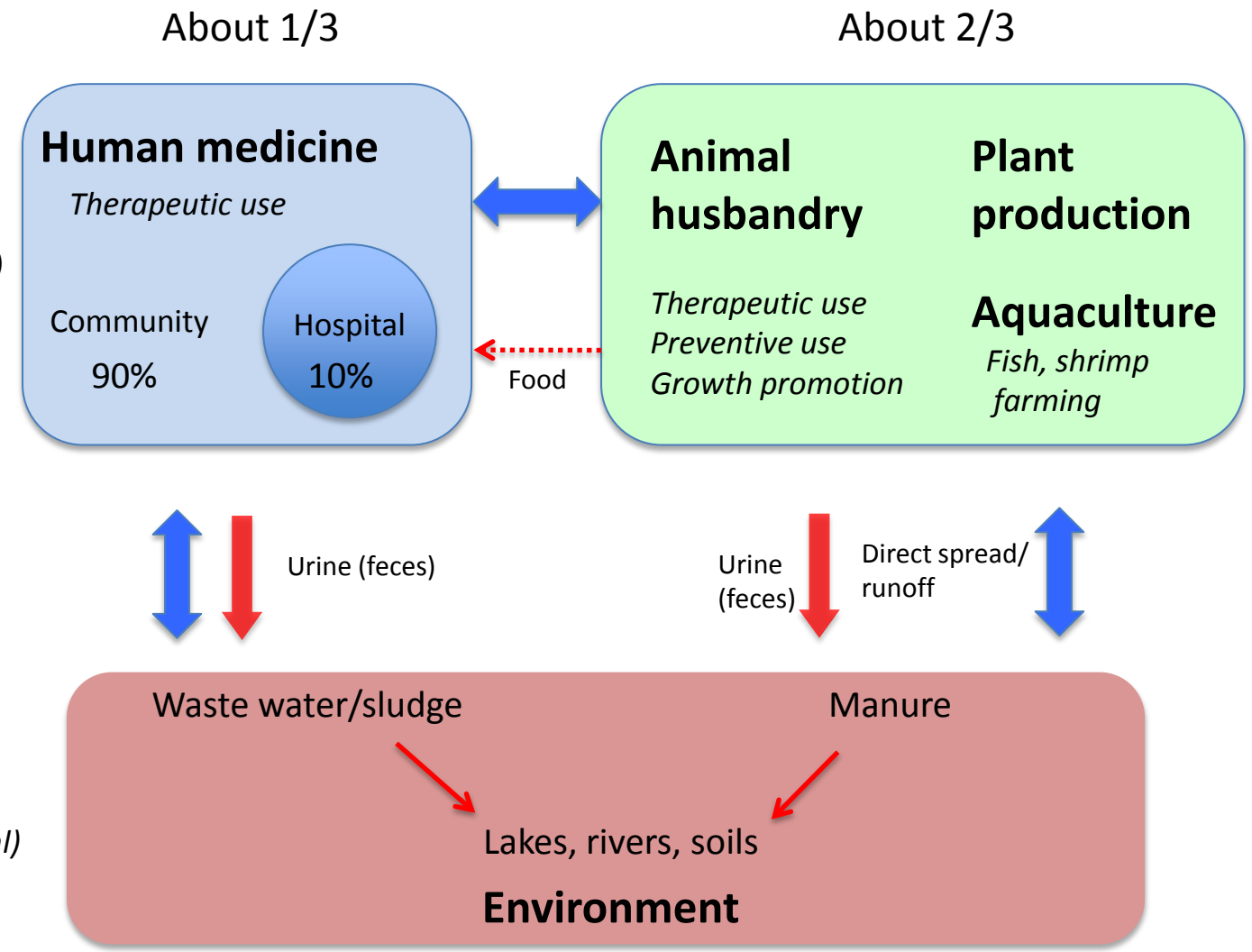
Selective pressures of varying strength in humans, animals and environment

Bacteria
Antibiotics

Both strong (ug/ml) AND weak (<<ug/ml) selective pressures



Weak selective pressures (ng-pg/ml)

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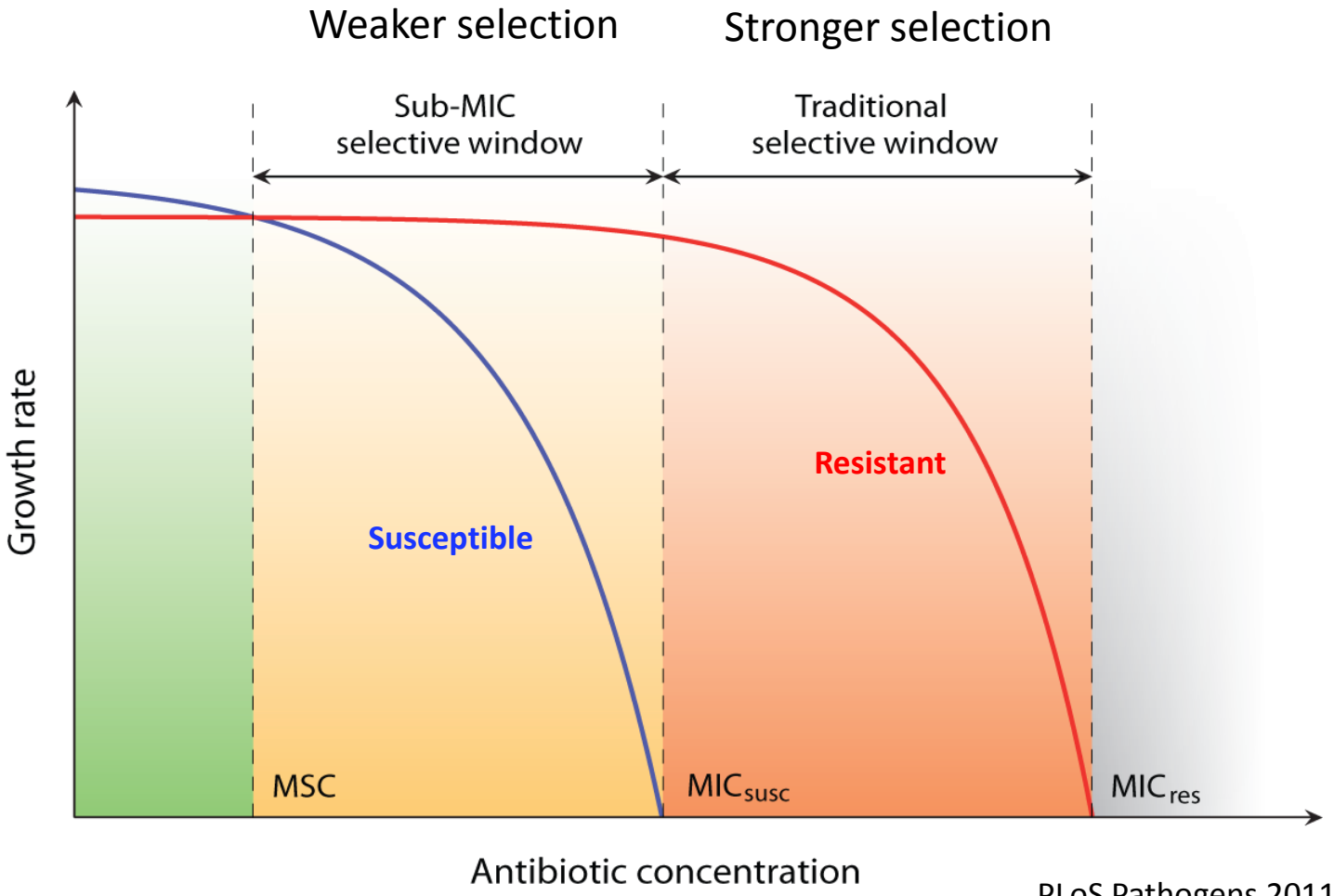
Key question: What are the lowest concentrations of antibiotics that are selective?

MIC= Minimal Inhibitory Concentration
MSC= Minimal Selective Concentration



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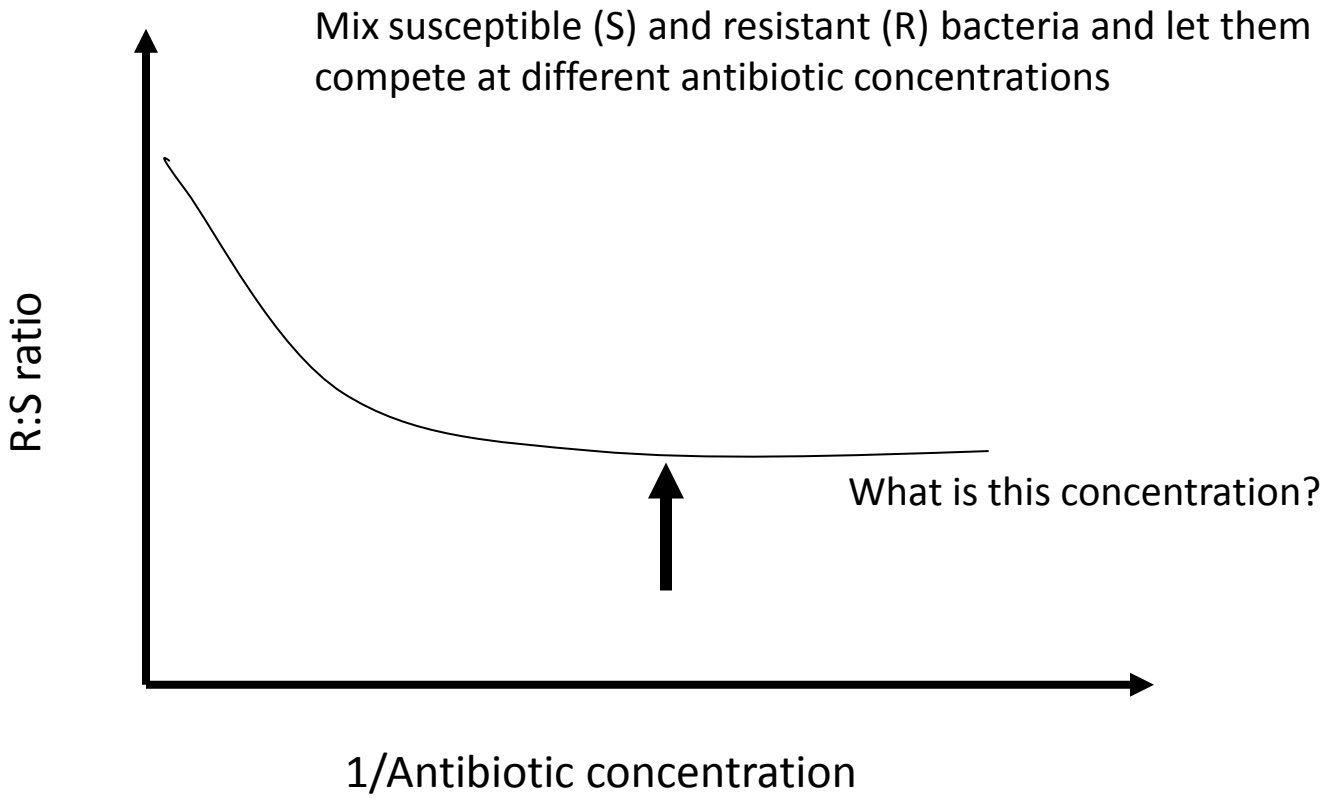
Key question: What are the lowest concentrations of antibiotics that are selective?



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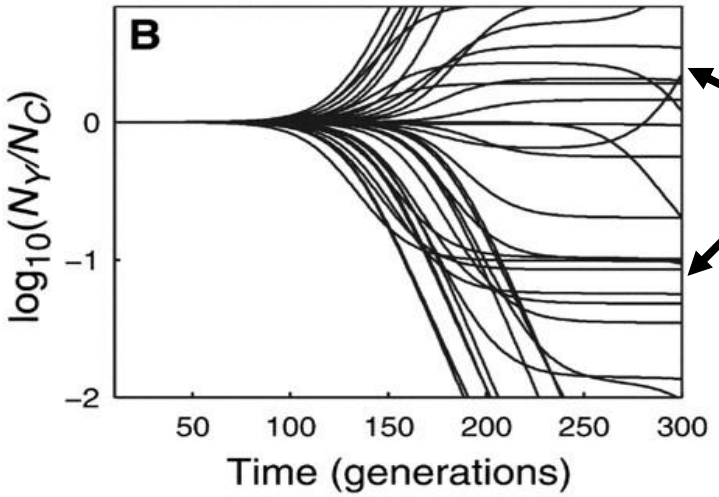
Determining the minimal selective concentration

Approach: Compete resistant and susceptible bacteria at different antibiotic concentrations to determine the lowest concentration at which resistant bacteria will be selected

Problem: Periodic selection will always limit sensitivity of competition assays



Take-over by adaptive mutation unrelated to competition set-up

Solutions:

1. Label cells with YFP/CFP and count with flow cytometer --> higher sensitivity
2. Pre-adapt strains--> decreases likelihood of adaptive mutations

Can detect Δs differences as small as 0.001 (0.1%)

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Sub-MIC selection with a fluoroquinolone

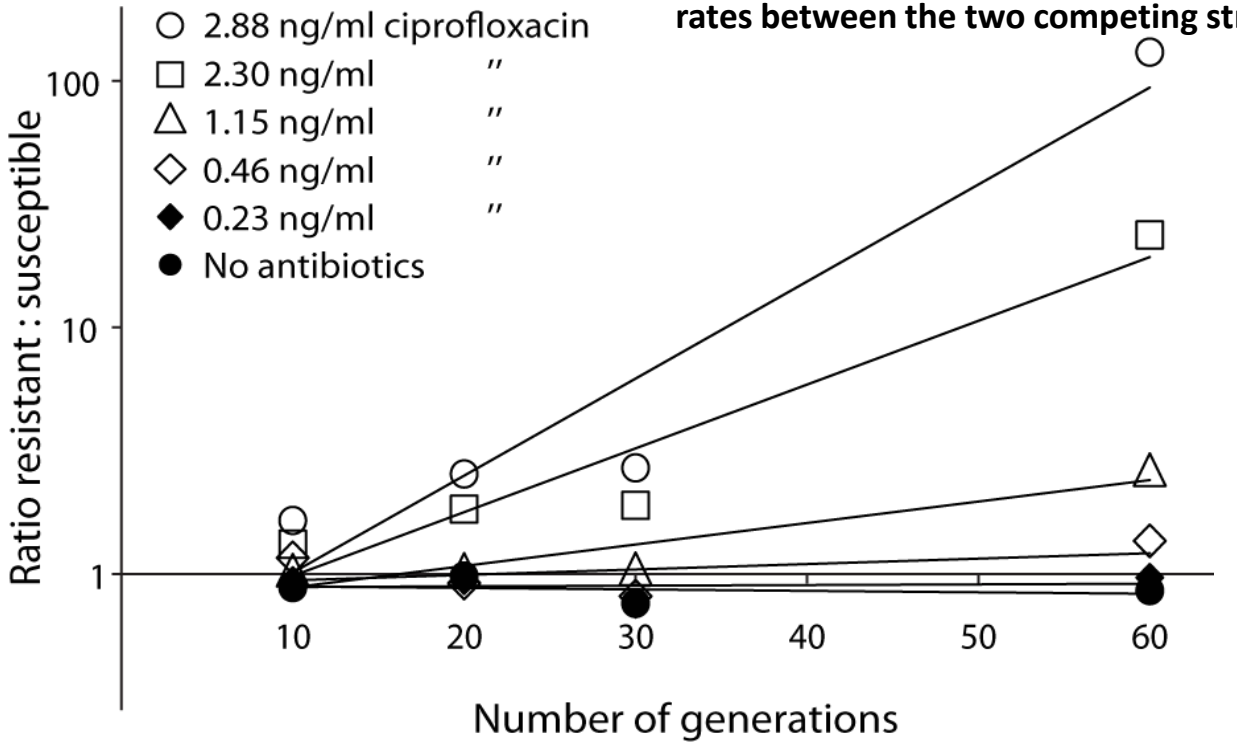
Competition at different antibiotic concentrations

R + S → Follow ratio by flow cytometry

CFP YFP

Pre-adapted strains

Slope of curves= difference in growth rates between the two competing strains



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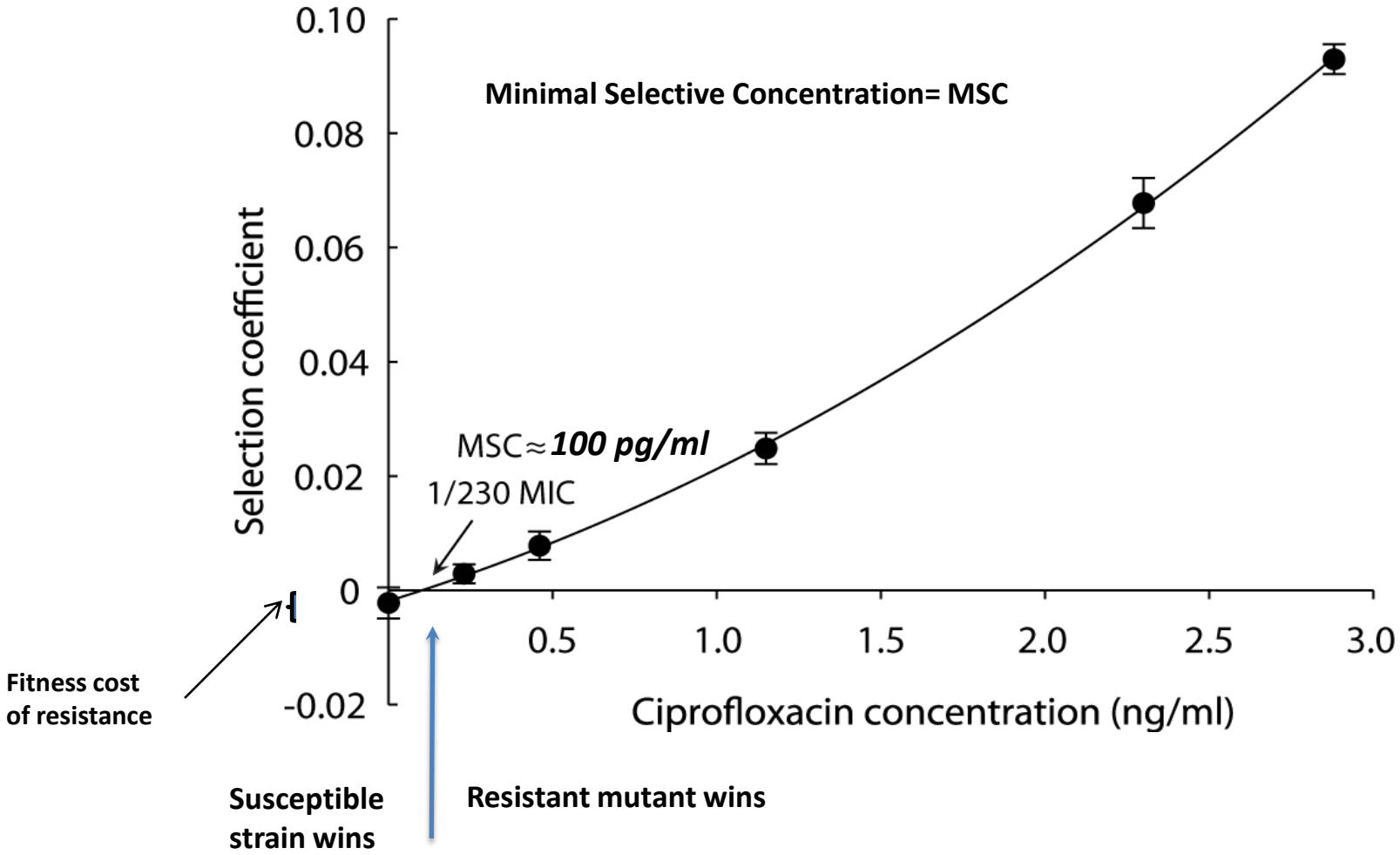
Sub-MIC selection



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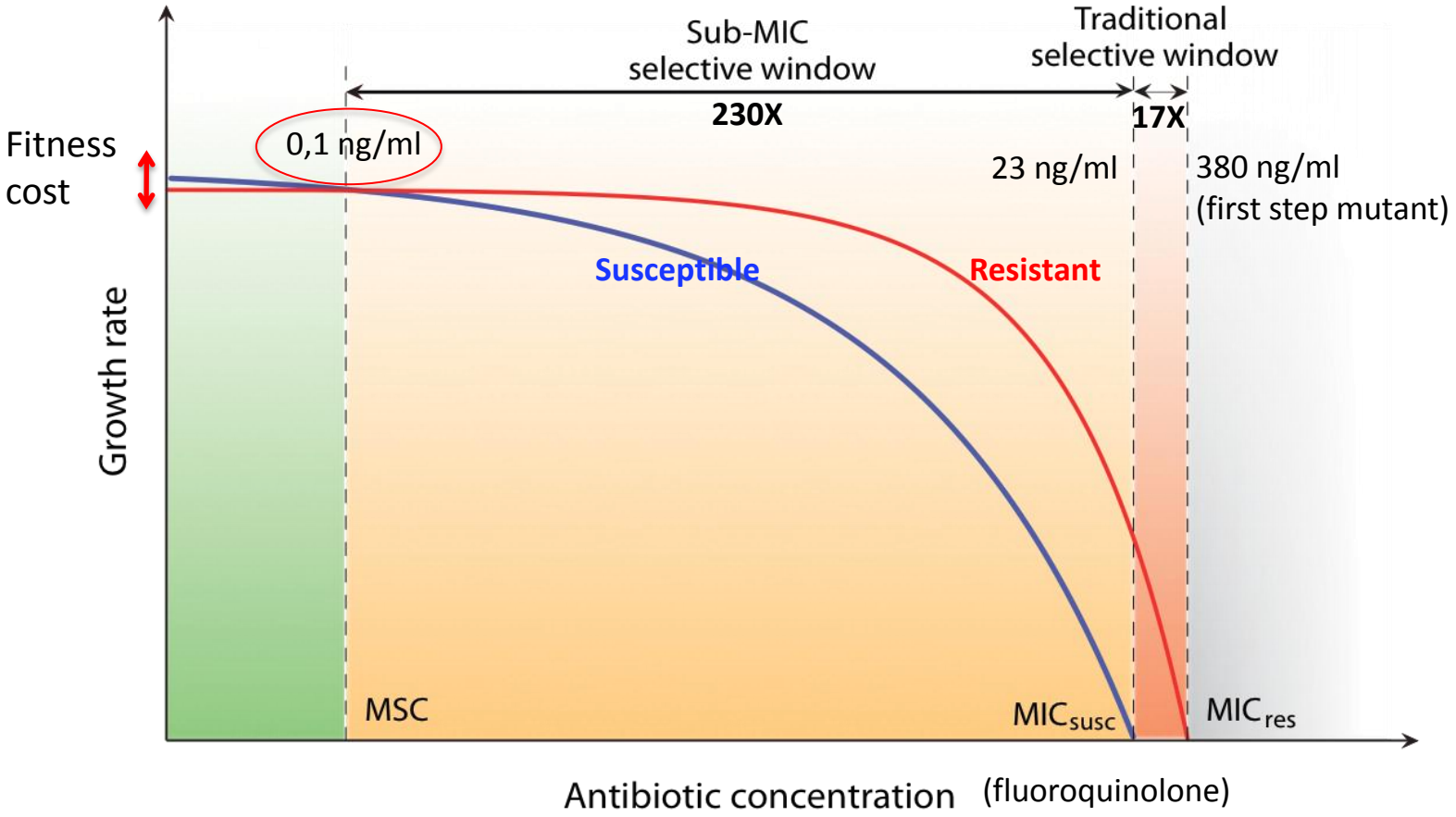
Sub-MIC selection

Sub-MIC selective window: ng-pg/ml, wider than traditional selective window



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How do antibiotic concentrations in the environment compare to determined MSCs?

Examples of concentrations of fluoroquinolones in water and sludge

Pharmaceutical industry sewage water Patancheru, India	31 ug per ml ***MSC in our experiments 0.1 ng/ml →*** ***310,000 –fold above selective concentration***
Sewage water from Uppsala University Hospital	2-14 ng per ml ***MSC in our experiments 0.1 ng/ml →*** ***20- to 140-fold above selective concentration***
Sludge (Sweden/US) Sewage water (Sweden)	0.1-48 ng per gram 0.1-0.3 ng per ml ***MSC in our experiments 0.1 ng/ml →*** ***1- to 480-fold above selective concentration***

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Selection of resistant *E. coli* in a semi-natural setting: Mallards in an experimental room

Mallards in the experimental room at National Veterinary Institute.

Uppsala

Intra-esophageal inoculation of a mix of resistant and susceptible bacteria.

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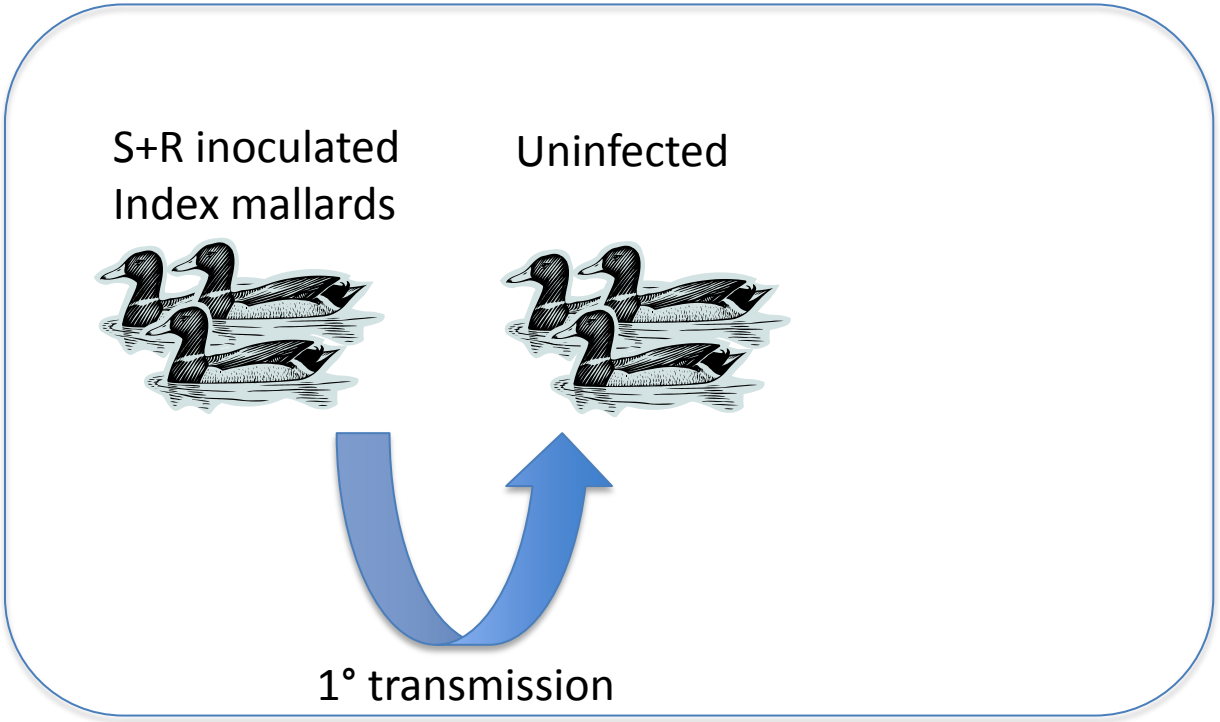
Transmission and selection experiment

Inoculate Mallards with fluoroquinolone - susceptible and – resistant E. coli

Introduce 3 index and 3 uninfected together, 1 day



Water with different levels of ciprofloxacin

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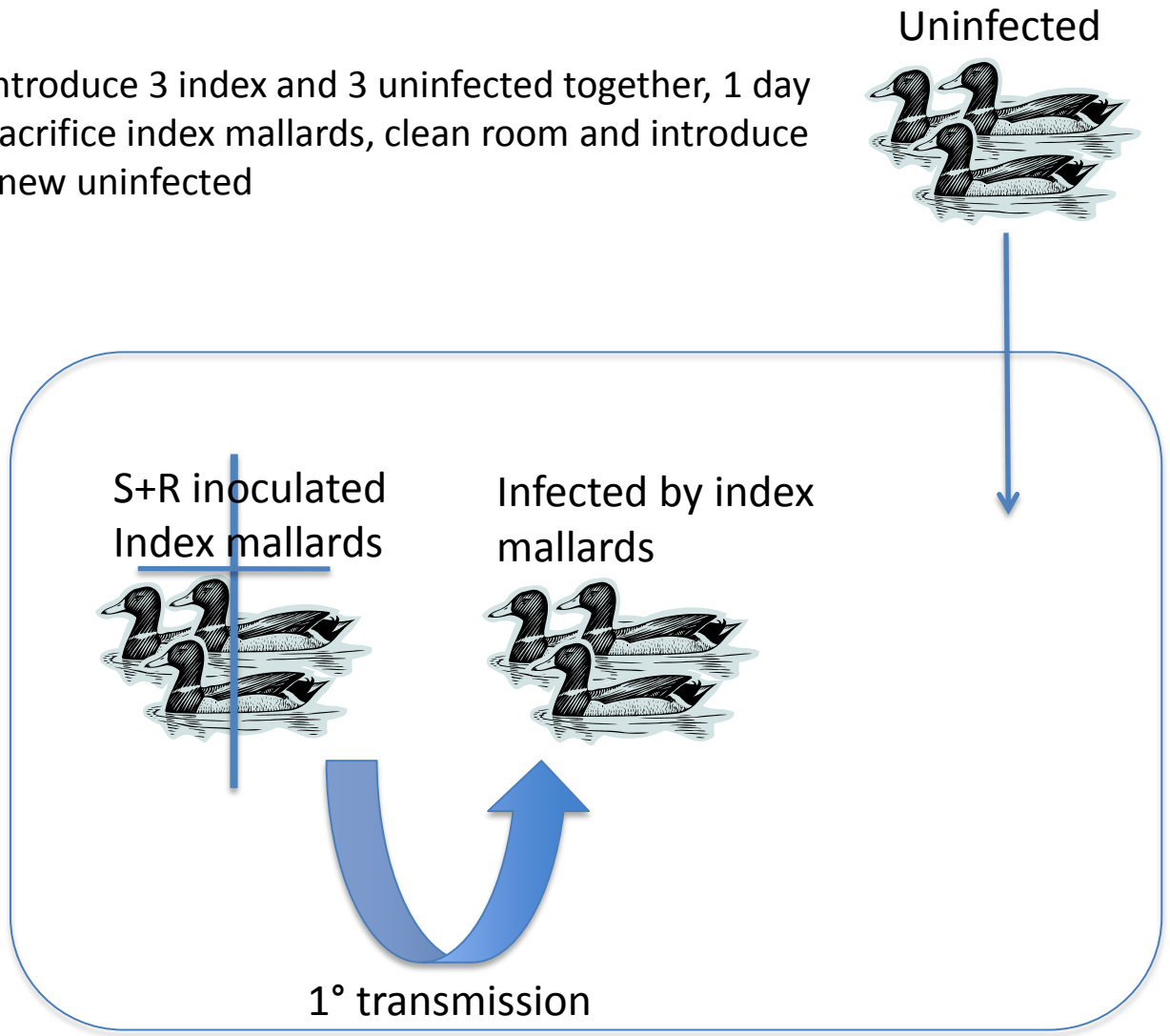
Transmission and selection experiment

Inoculate Mallards with fluoroquinolone susceptible and –resistant E. coli

-Introduce 3 index and 3 uninfected together, 1 day
-Sacrifice index mallards, clean room and introduce 3 new uninfected



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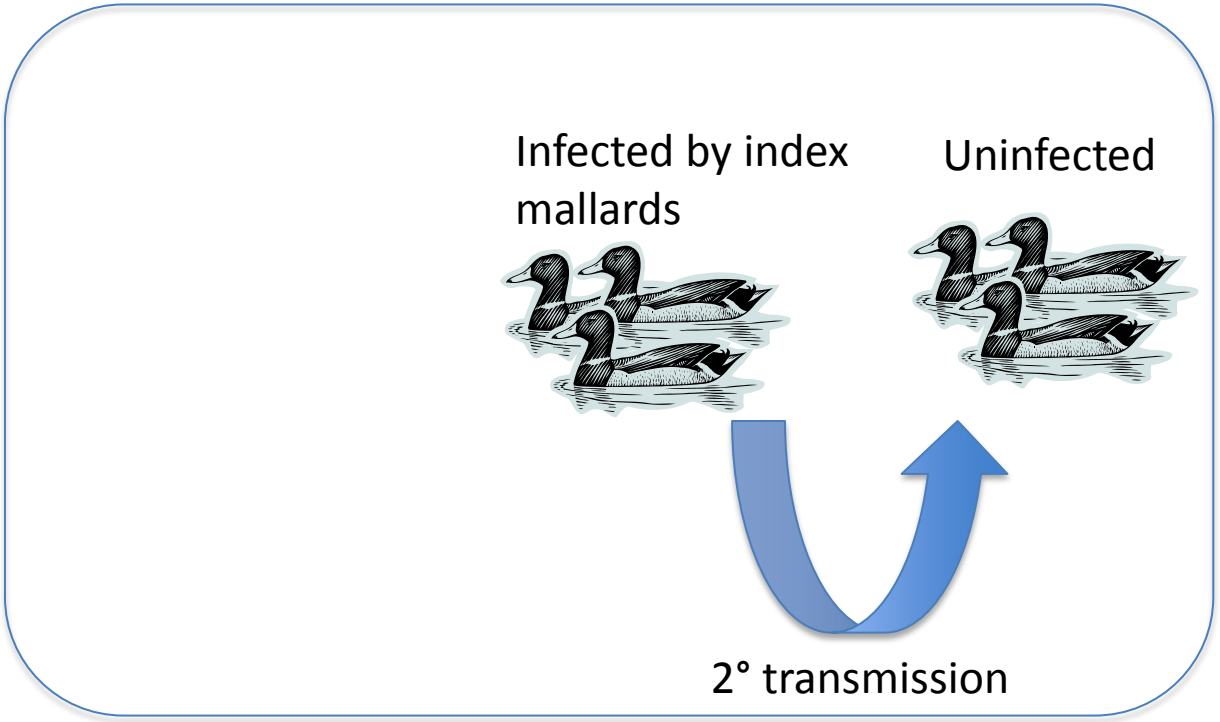
Transmission and selection experiment

Inoculate Mallards with fluoroquinolone susceptible and –resistant E. coli

-Introduce 3 index and 3 uninfected together, 1 day
-Sacrifice index mallards, clean room and introduce 3 new uninfected
-Sample feces of all birds on day 1, 2, 3, 4, 6, 8 and 11 for presence of susceptible and resistant *E. coli*



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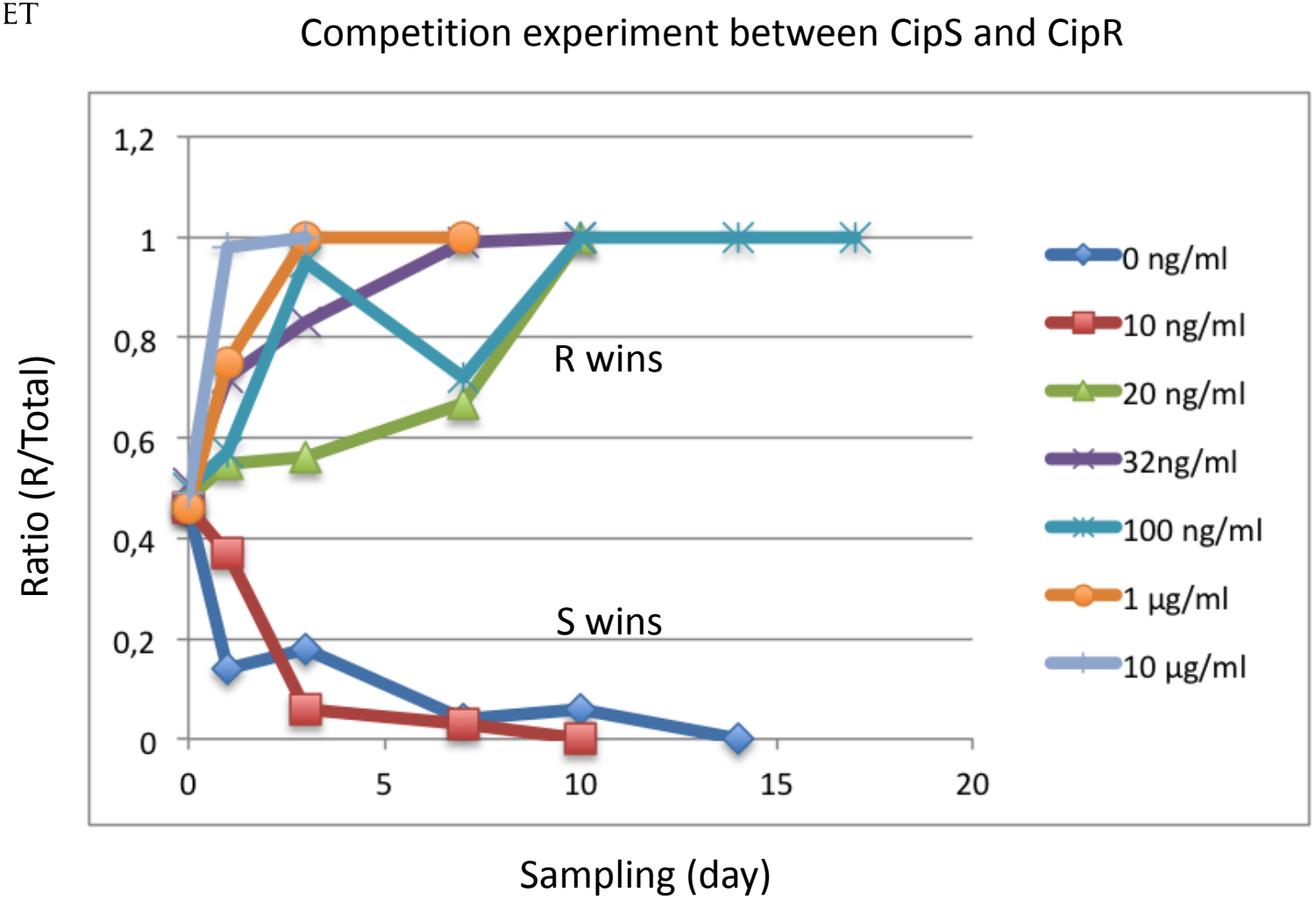
Selection for ciprofloxacin-resistant *E. coli* in Mallards



MSC of ciprofloxacin in pond water about 1 ng/ml (10x what is seen in vitro).

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Selection for a costly multi-resistance plasmid by presence of heavy metals/antibiotics at low levels

Found in ESBL-producing *Klebsiella pneumoniae* clone at Uppsala University Hospital.

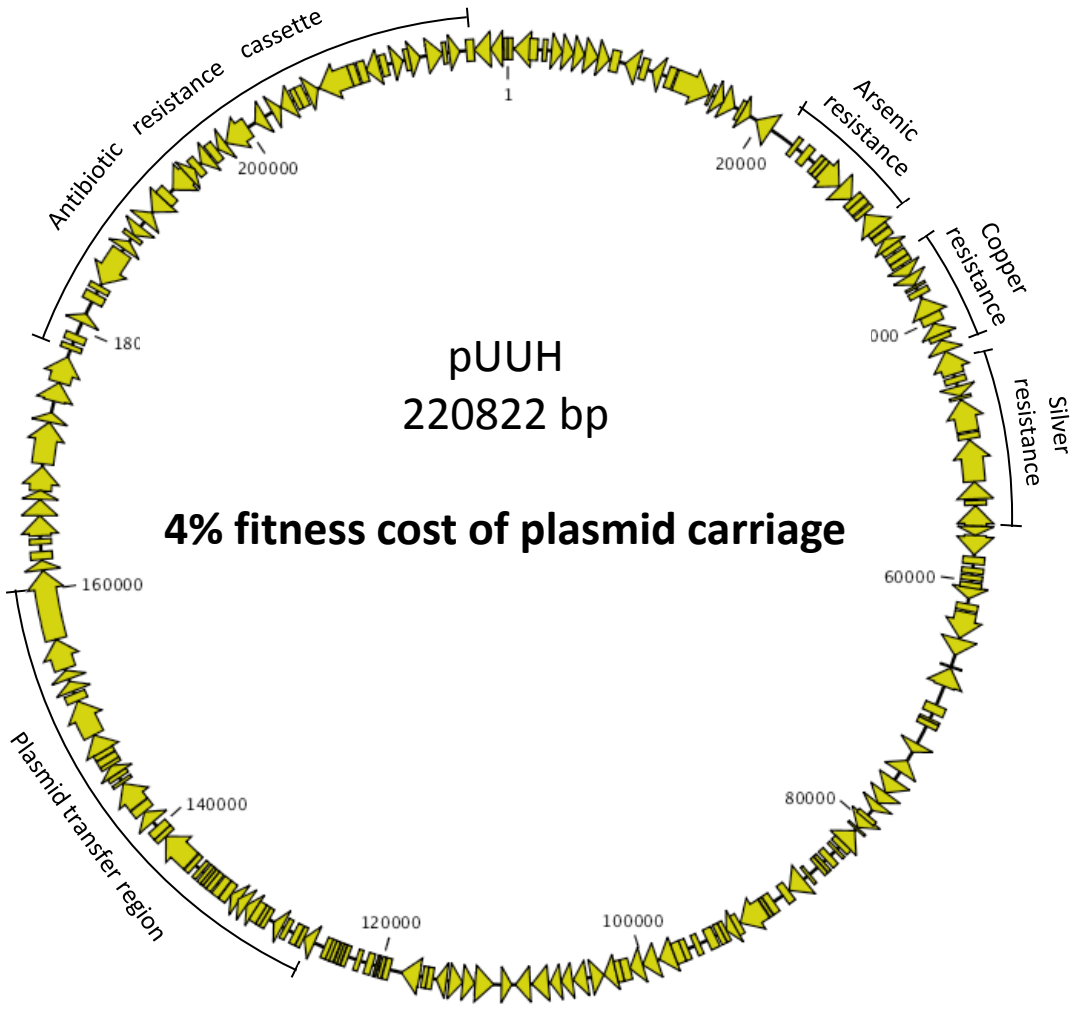
Large conjugative plasmid

225 genes, 20% involved in resistance to:
β-lactams, Aminoglycosides, Trimethoprim, Sulphonamides, Macrolides, Tetracycline
Arsenic, Copper, Silver

MSC for this plasmid:

Arsenic: 6 ug/ml (200x below MIC)
Roxarsone/nitarsone used in poultry/swine production, about 6x above MSC in feed
Copper: 0.08 ug/ml (15x below MIC)
Silver: 0.25 ug/ml (12x below MIC)

Tetracycline: 200 ng/ml (10x below MIC)
Trimethoprim: 30 ng/ml (60x below MIC)



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Selection at low, non-lethal drug concentrations (as compared to high, lethal concentrations)

1. **Higher rates of emergence of resistant mutants**

   a. Selection for common mutations of small effect
   b. Mutations can form during growth after selection is applied
   c. Selective agent can modulate rates of mutation, recombination, and horizontal gene transfer

2. **Selection for more problematic types of resistant mutants**

   a. Strong enrichment for mutator bacteria with faster resistance development
   b. Mutants with higher fitness selected

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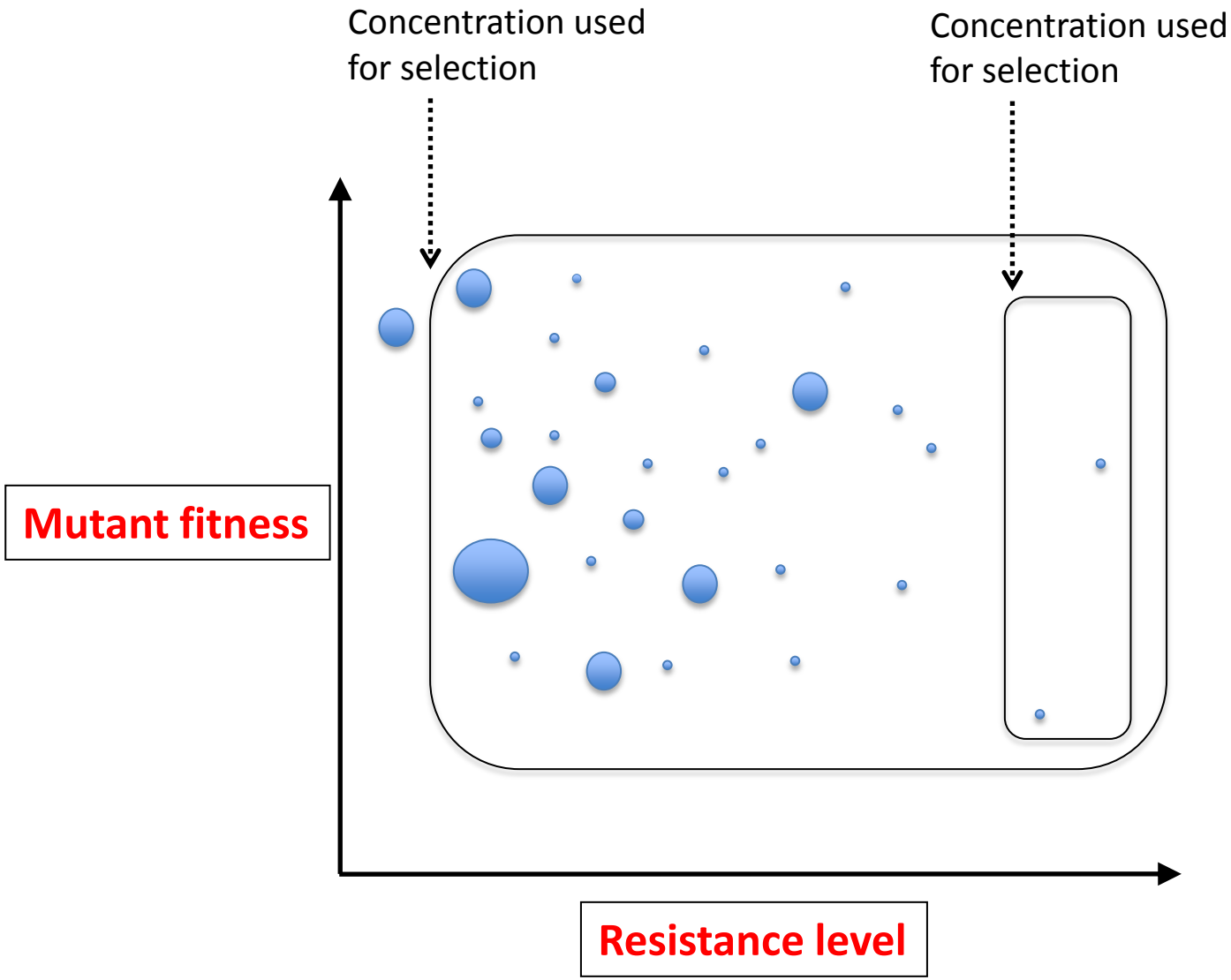
1. Higher rates of emergence of resistant mutants

a. Selection for common mutations of small effect



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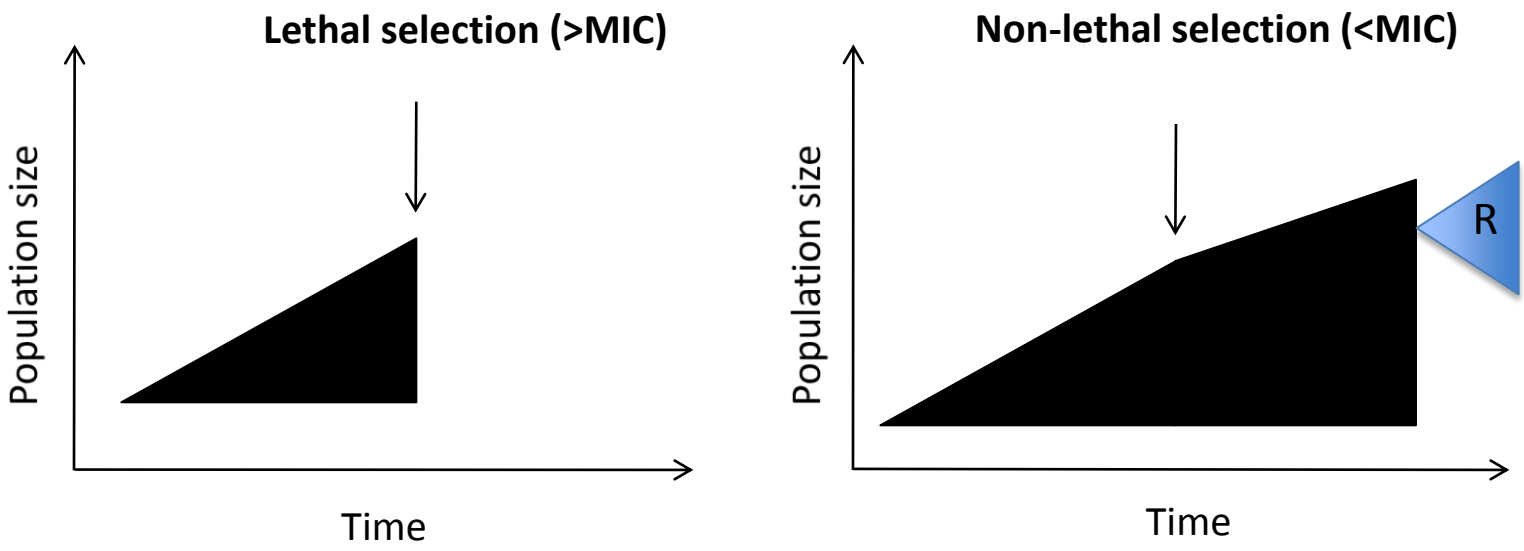
1. Higher rates of emergence of resistant mutants

b. Mutations can form during growth after selection is applied



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1. Higher rates of emergence of resistant mutants

c. Selective agent can modulate rates of mutation, recombination, and horizontal gene transfer

Fluoroquinolones:

SOS induction→ increased mutation rates
increased rates of recombination (ICE, integrons)

Oxygen radicals→ increased mutation rates

β-lactams:

SOS induction→ increased mutation rates

Aminoglycosides:

Translational misreading→ increased mutation rates

Tetracycline:

Stimulates horizontal gene transfer of conjugative elements

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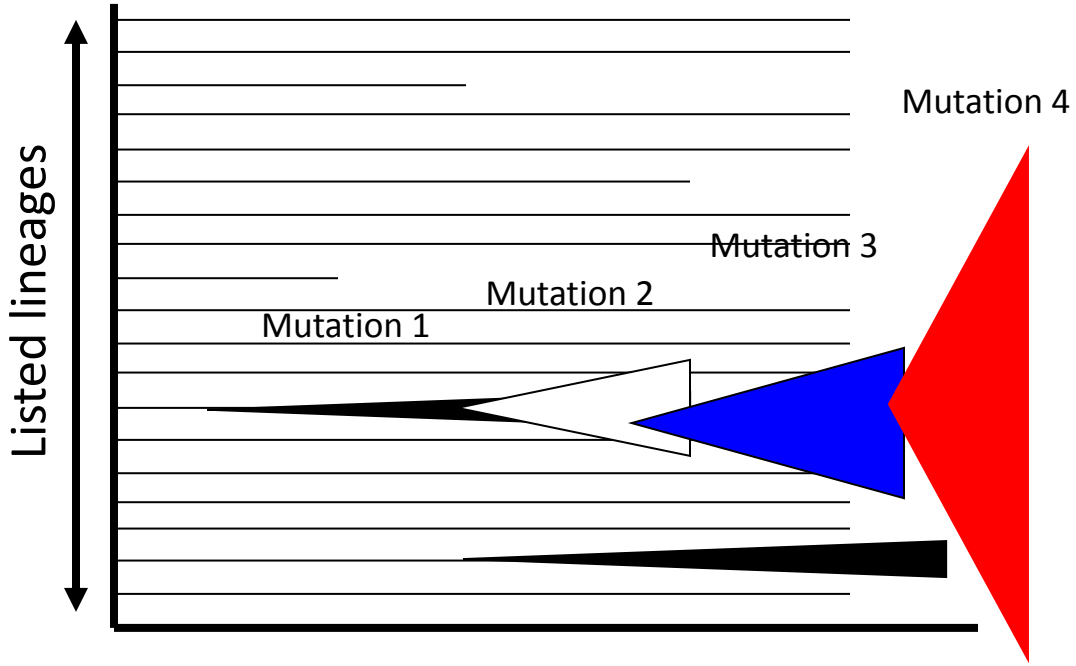
2. Selection for more problematic types of resistant mutants

a. Strong enrichment for mutator bacteria

Mutator bacteria have defective DNA repair and higher mutation rates→ Higher risk of further resistance development

Line thickness indicates lineage size



Four successive mutations → enrichment for mutators at each step

e.g. if frequency of mutators is 10^{-5} at start and the mutator has a 100-fold higher mutation rate→ majority of cells are mutators after successive selection for a few mutations (Mao et al 1997, J Bact)

At mutation-selection balance:
Frequency = Mutation rate/Selection coefficient = $10^{-7}/10^{-2} = 10^{-5}$

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2. Selection for more problematic types of resistant mutants

a. Strong enrichment for mutator bacteria

Example: Streptomycin resistance in *Salmonella typhimurium*

High streptomycin concentration (10x MIC)	*rpsL* (S12) mutations
No mutators	
Low streptomycin concentration (0.1xMIC)	*gidB, malT* mutations *leuV, leuQ, serX* tRNA genes + other mutations
60% mutators	

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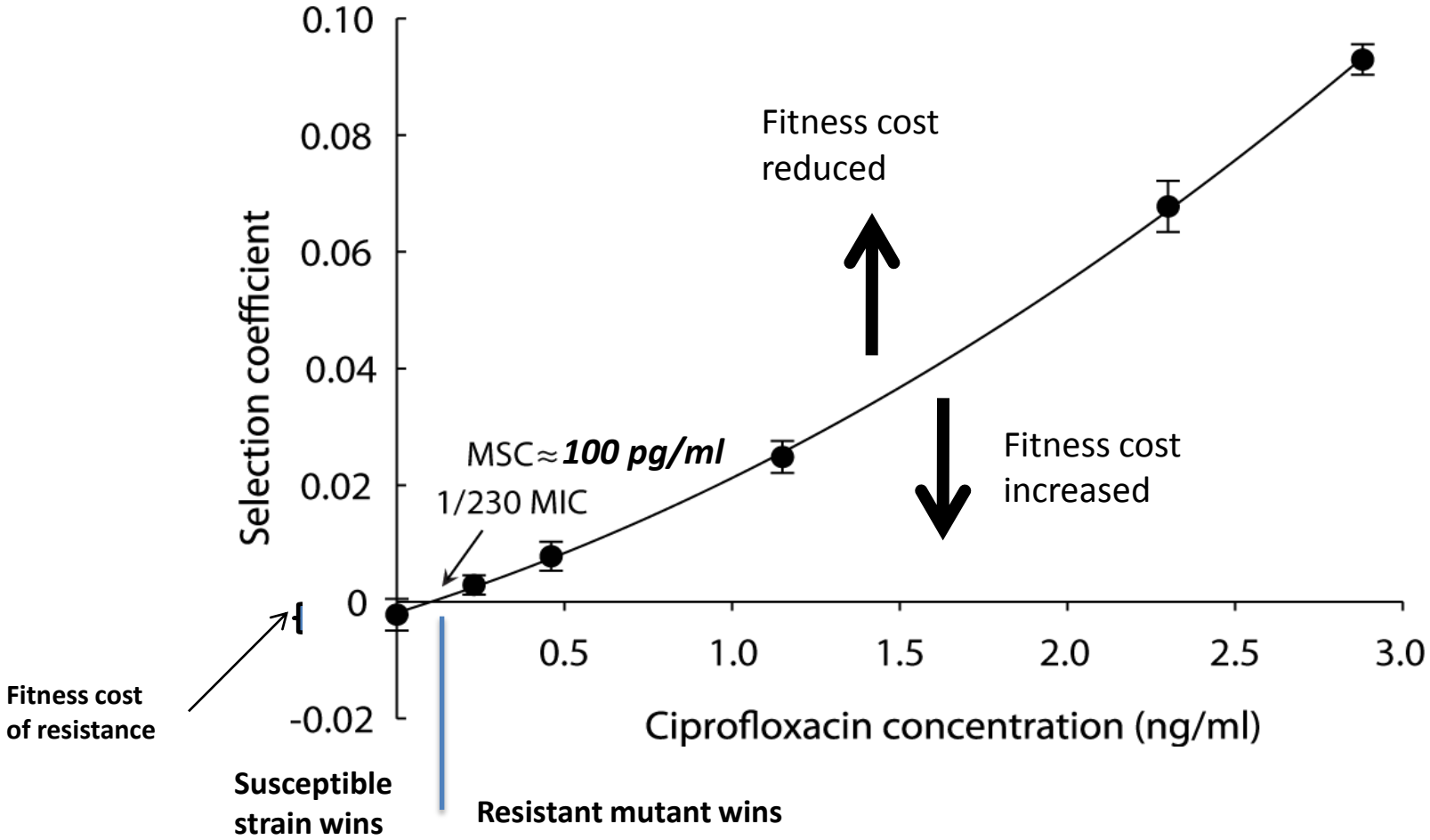
2. Selection for more problematic types of resistant mutants

b. Mutants with higher fitness selected



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2. Selection for more problematic types of resistant mutants

b. Mutants with higher fitness selected

Example: Streptomycin resistance in *Salmonella typhimurium*

High streptomycin concentration (10x MIC)	*rpsL* (S12) mutations
No mutators	High resistance (>1024 mg/L) High fitness cost (3-30%)
Low streptomycin concentration (0.1xMIC)	*gidB, malT* mutations *leuV, leuQ, serX* tRNA genes
60% mutators	High resistance (> 1024mg/L) due to combination of many mutations of small effect Low fitness cost (<2%)

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Summary (1)

1. Very low antibiotic levels (ng-pg/ml) can select for high-level antibiotic resistance

2. These low antibiotic concentrations are present in many environments and in humans/animals during treatment and growth promotion

3. Need to reduce levels of antibiotic residues in environment
   - -discontinue growth promotion use of antibiotics
   - -ozon treatment of waste water (efficient, relatively cheap)

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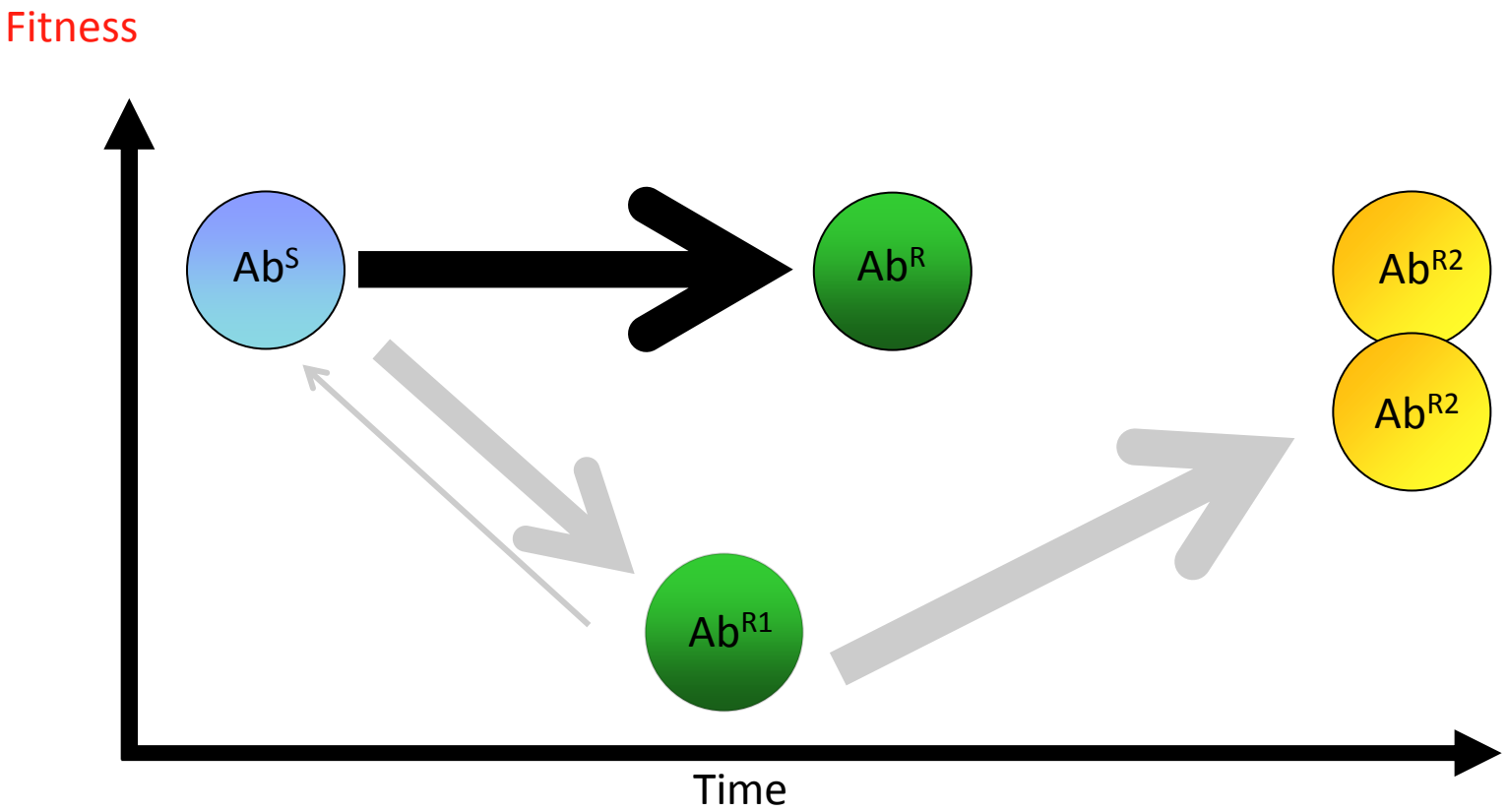
Summary (2)

4. Selections at <MIC have different trajectories and endpoints than selection >MIC and mutants selected at sub-MIC are potentially more problematic:

-faster emergence
-enrichment for mutators
-selection for higher fitness resistant mutants



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Vetenskapsrådet

SEVENTH FRAMEWORK PROGRAMME

EvoTAR
Evolution and Transfer of Antibiotic Resistance

Ribo CORE Consortium of Research and Education

Forskningsrådet Formas
Formas främjar framstående forskning för hållbar utveckling

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The commonest mutation types of all are copy number variations:

Duplication and amplifications

(also plasmid copy number variants in bacteria
and extra chromosomes (aneuploidy) in eukaryotes

Duplications are:

-Deleterious alone (non-selective conditions)
-Held at steady state frequency in population (without selection)
-Can provide a beneficial phenotype under selection

Some recent papers to read for those with expanding minds:

Nilsson et al 2006 PNAS
Andersson and Hughes 2009 Ann Rev Genet
Sandegren and Andersson 2009 Nat Rev Microbiol
Lind et al 2010 Mol Microbiol
Pränting and Andersson 2011 Mol Microbiol
Roth and Andersson 2012 Cell
Näslund et al 2012 Science
Adler et al 2014 Mol Biol Evol

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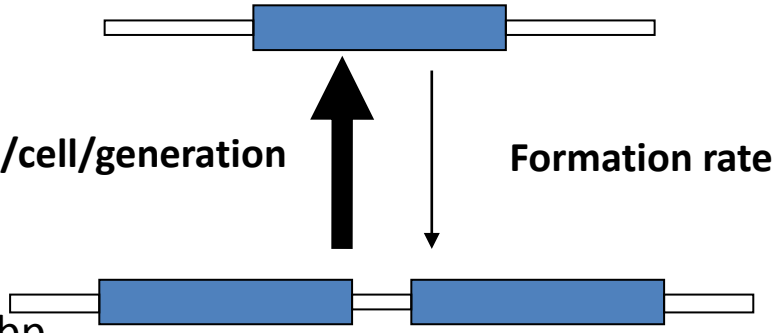
Gene duplication-amplification



Loss rate: 10^{-3} to 10^{-1}/cell/generation

Formation rate: 10^{-5} to 10^{-3}/cell/generation

Amplified region can
be <1kbp up to several Mbp

Steady-state frequencies of duplications vary from 0.03 to 10^{-5} (typically around 10^{-3}) depending on gene/region

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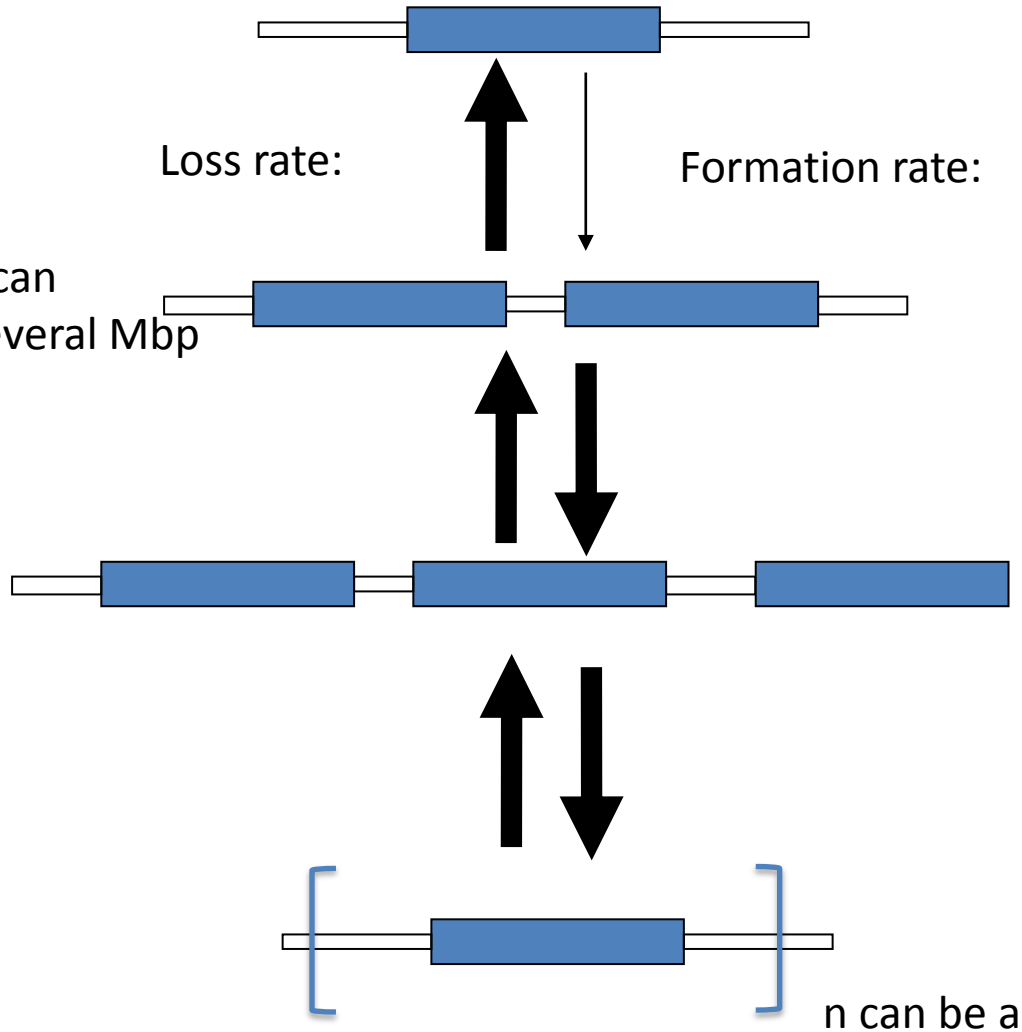
Gene duplication-amplification



Amplified region can be <1kbp up to several Mbp

Selection for function inside duplication can rapidly drive up copy number

We have observed cases with 100 copies of a 60 kbp region→ 6Mbp of extra DNA

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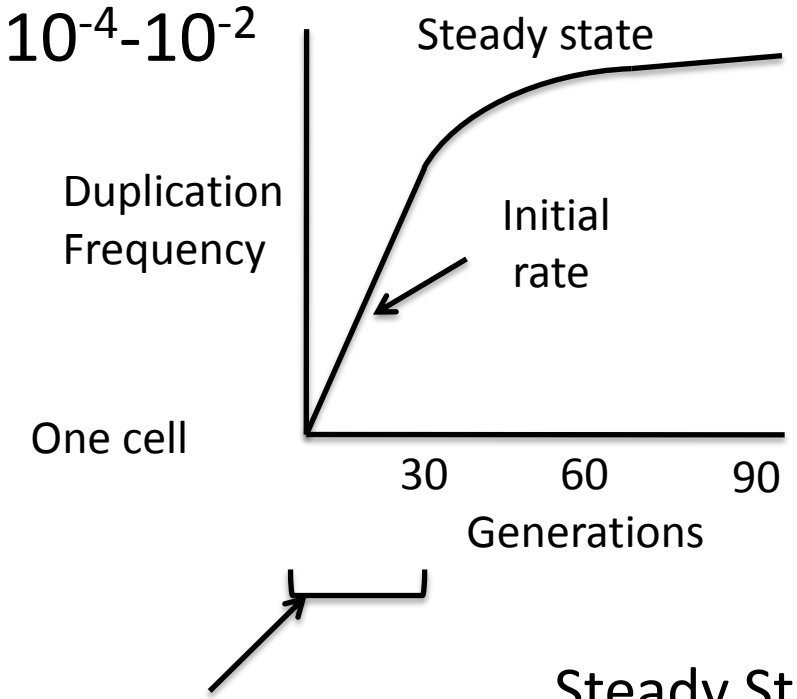
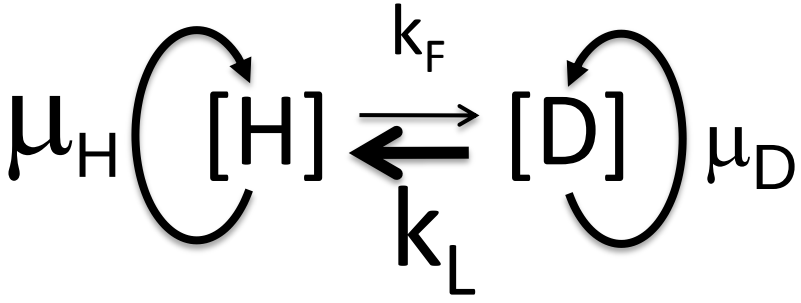
Duplication frequency comes to high steady state without selection





Steady State Duplication Frequency (approximation)

$$\frac{D}{H} = \frac{k_F}{k_L + \left(\frac{\mu_H - \mu_D}{\mu_H}\right)}$$

Reams et al 2010 Genetics

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Tandem gene amplifications are very different from other mutations

1. High frequency

   10^{-5} to 10^{-2} per cell per gene, ca. 20% of cells in a population have a duplication some where at any given time → huge standing genetic variation → essentially no waiting time for an amplification to appear

   Compare point mutation where frequency is 10^{5} to 10^{7} lower

   Idea of genetic homogeneity in a small population largely correct with regard to SNPs but incorrect with regard to rearrangements

2. Unstable = Rapidly reversible

   Rate of mutational reversibility for a point mutation is 10^{-10} to 10^{-12}/ base pair/generation whereas for a duplication it can be as high as 0.15/cell/generation, i.e. almost as fast as a standard regulatory mechanism

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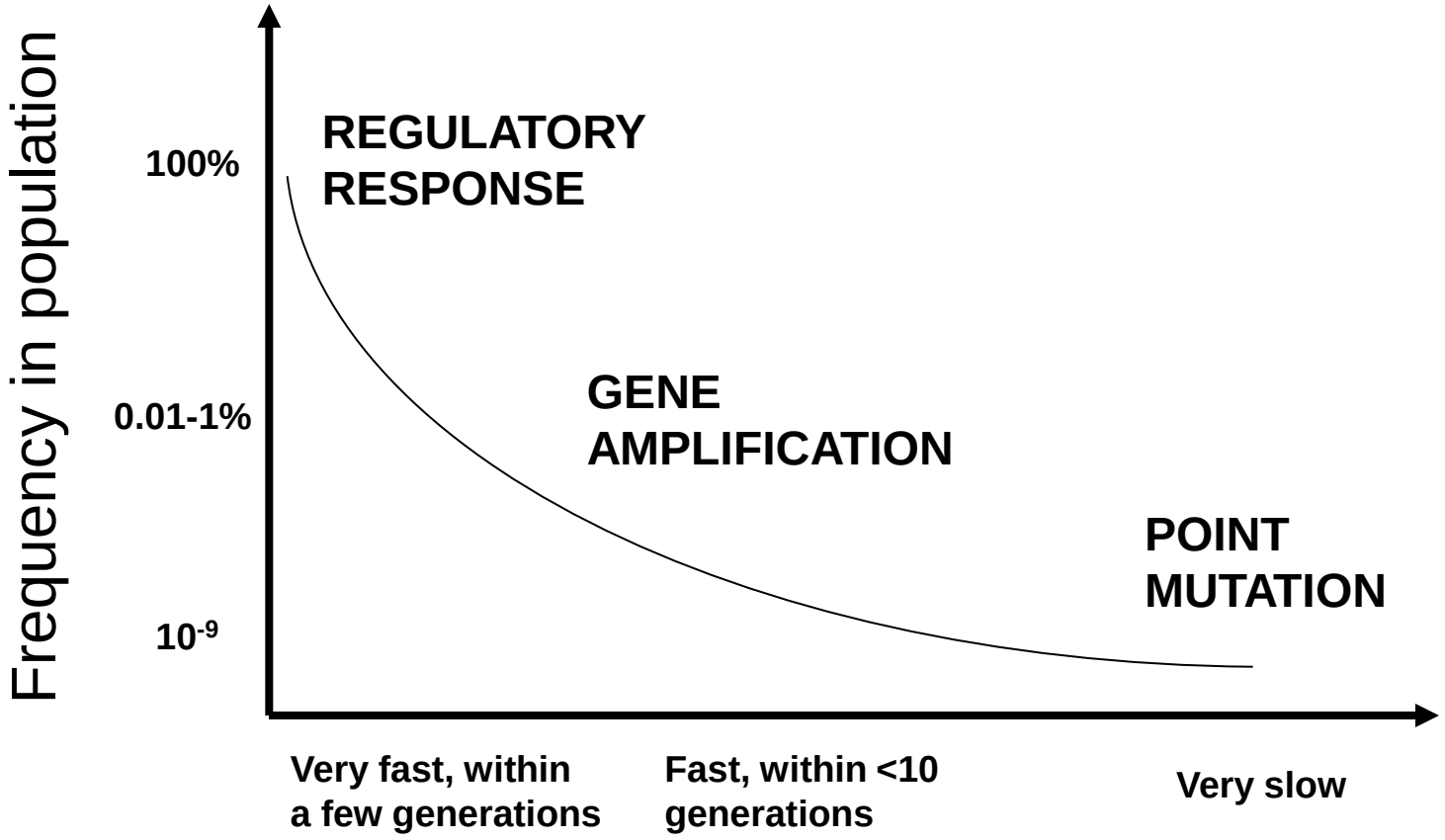
Gene amplification—more similar to regulatory mechanisms than classical stable mutations



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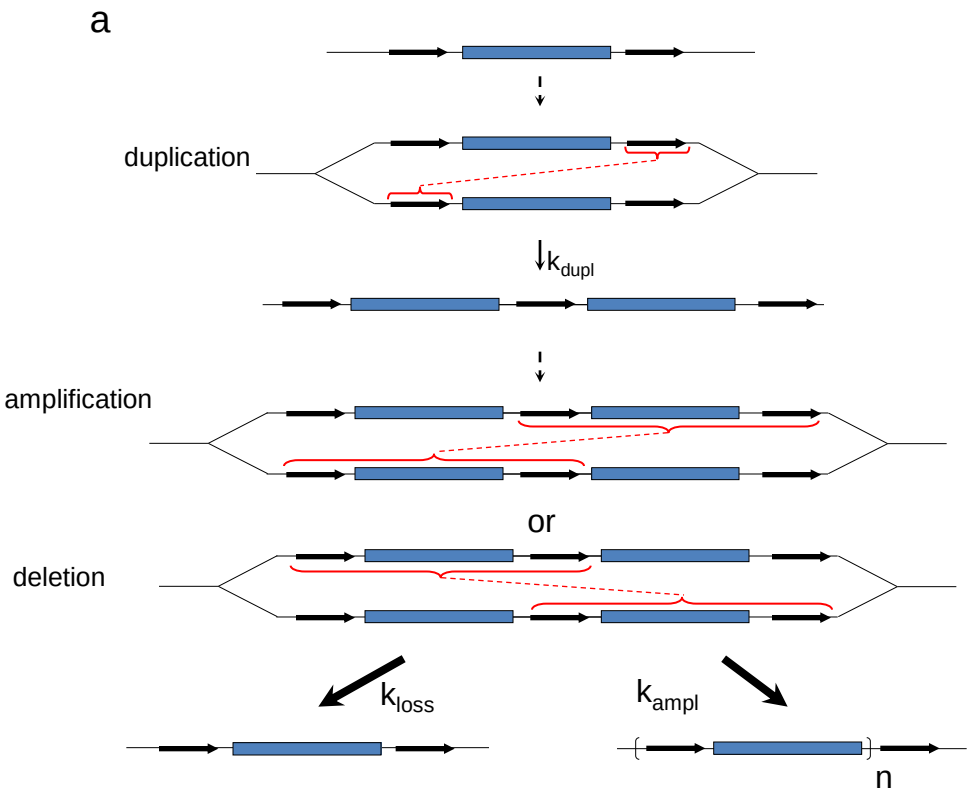
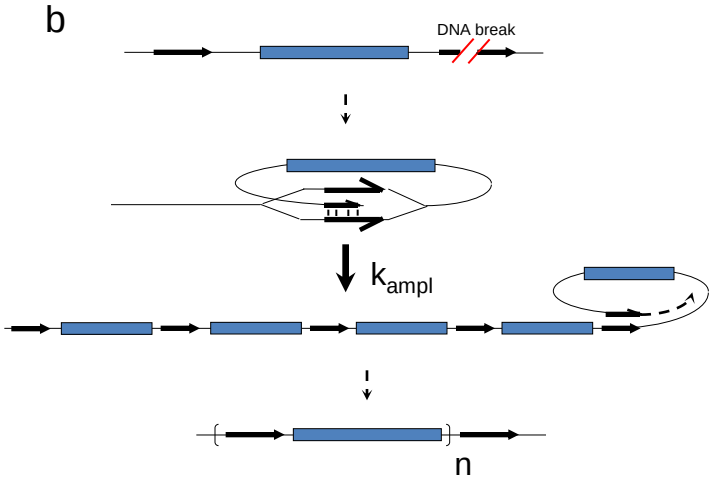
How do duplications and amplifications form?

Non-reciprocal recombination



Rolling-circle mechanism



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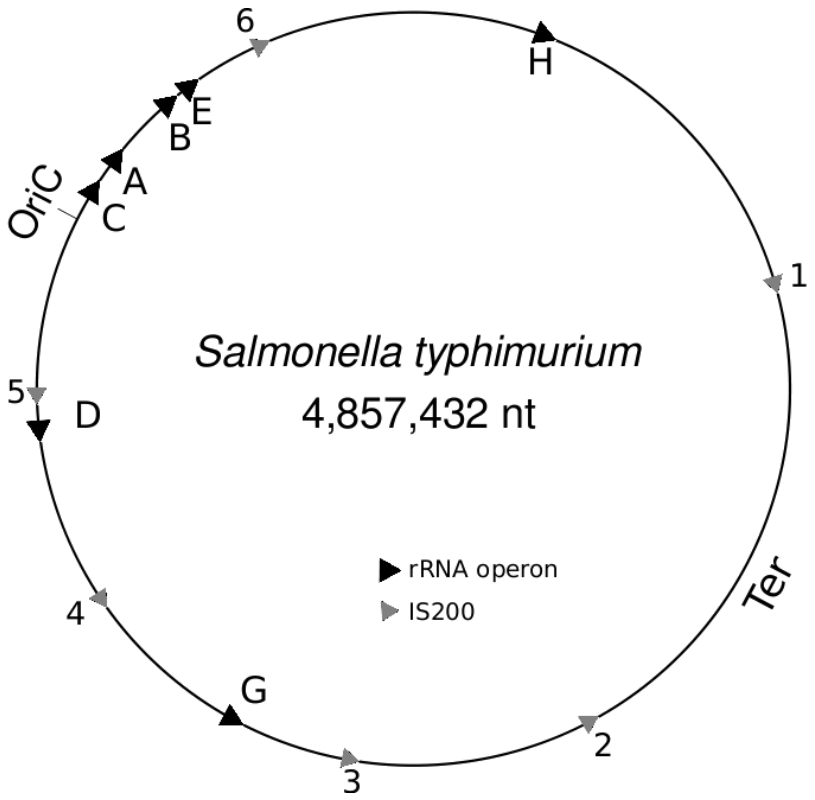
Direct sequence repeats in *Salmonella typhimurium*



Direct repeats often involved (but not always):

7 rRNA operons (~5kb with almost 100% identity)

Duplications ~40kb to ~1Mb

6 IS*200* elements (709 bp perfect identity)

Duplications ~400kb to ~1.3 Mb

~500 REP (repetetive extragenic palindromes) / BIME (bacterial interspersed mosaic elements) sequences (~40 to ~500 bp imperfect identity)

Varying sizes of duplications

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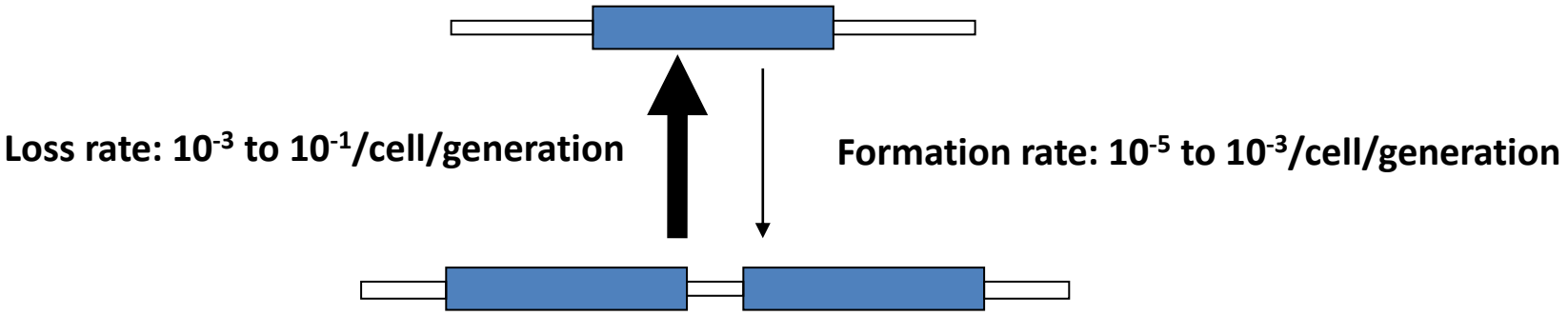
Determining duplication frequencies, formation rates and loss rates



Steady-state frequencies of duplications vary from 0.03 to 10^{-4} (typically around 10^{-3}) depending on gene/region

If the duplication is neutral, the steady-state frequency is the ratio of the formation rate/loss rate

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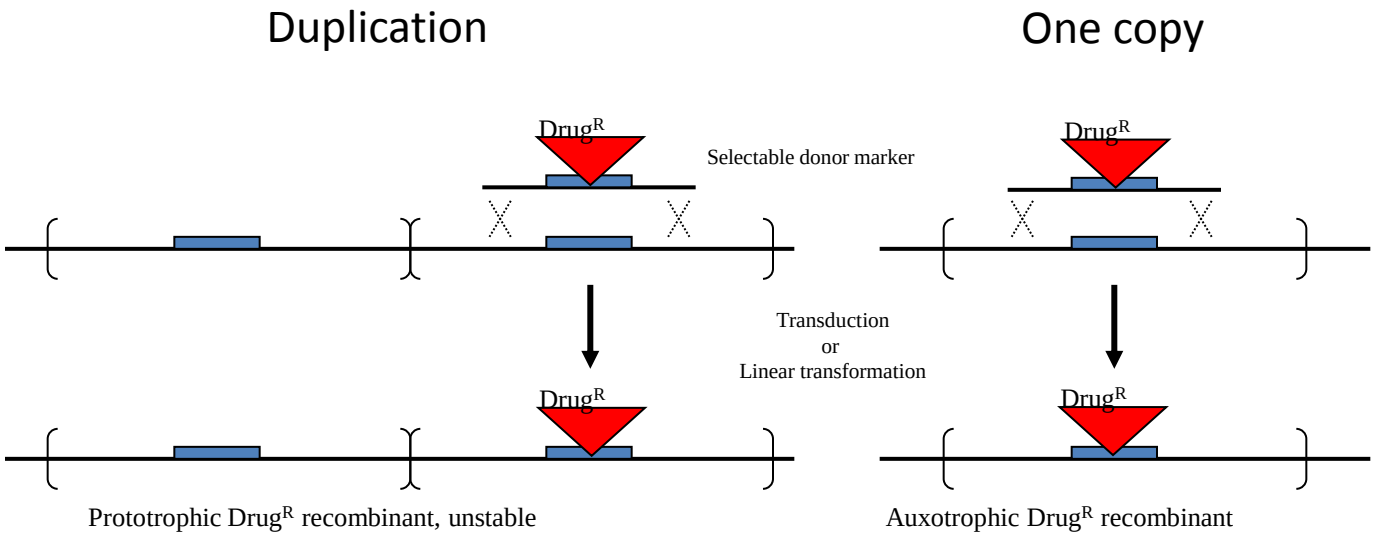
Assay to determine Frequency of Duplication of a gene



Duplication frequency= # prototrophic drugR recombinants/total # of recombinants

Transduction and linear transformation assay for duplications. Linear DNA fragment (a transducing fragment or a PCR-generated linear fragment) carries a drug resistance gene inactivating a biosynthetic gene. Selection for drug resistant recombinants (after phage-mediated transduction or linear transformation) results in inactivation of the chromosomal copy of the biosynthetic gene. If the gene is unique the strain will be auxotrophic. If the gene was present if two or more copies the strain will be drug resistant but remain prototrophic.

Anderson and Roth (1979) PNAS

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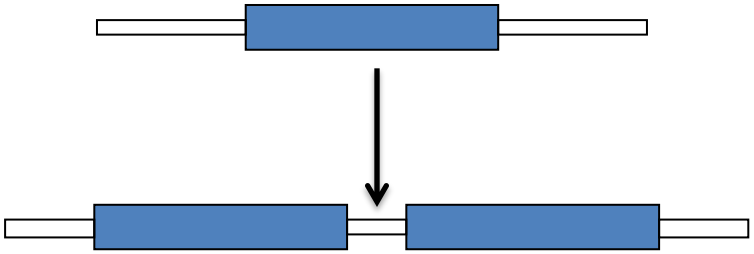
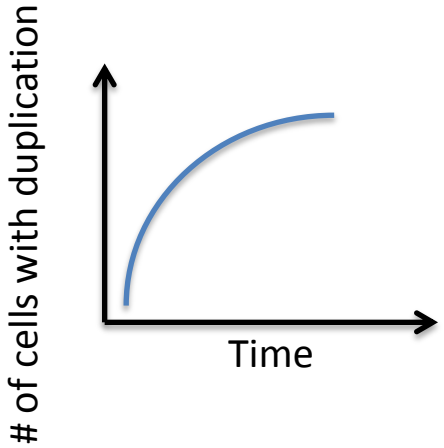
Assay to determine Formation Rate of a duplication





1. Start a culture from a few cells (lacking duplication)

2. Grow and sample over time by using previous assay

3. Follow increase in frequency of duplication

4. Initial rate of increase is formation rate

Reams et al 2010 Genetics

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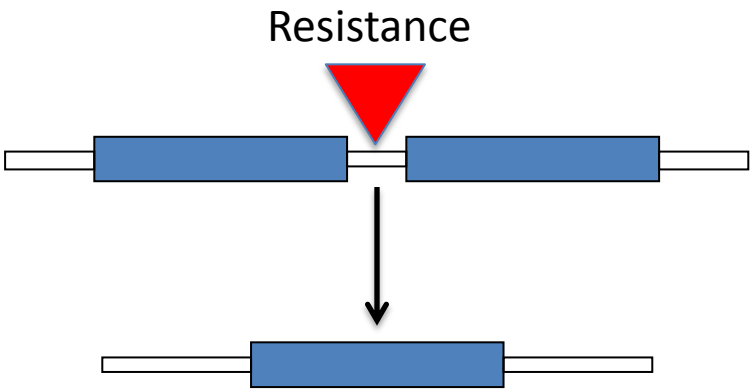
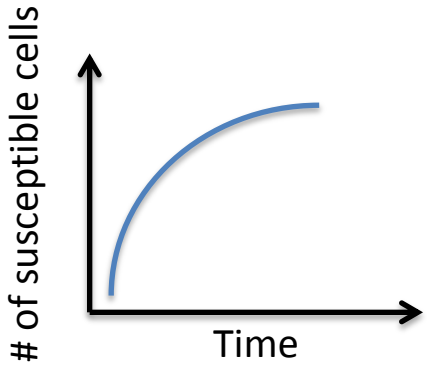
Assay to determine Loss Rate of a duplication





1. Construct a duplication of the region of interest with a resistance marker at the join point. Can maintain duplication by selection for resistance marker.

2. Remove antibiotic selection and follow increase in frequency of one copy state by loss of resistance marker.

3. Rate of increase in frequency of susceptible cells is the loss rate.

Adler et al 2014 Mol Biol Evol

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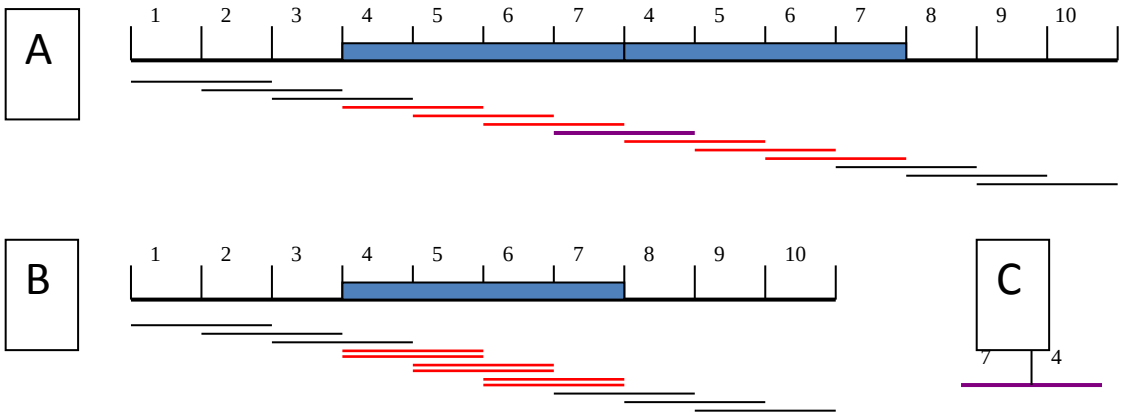
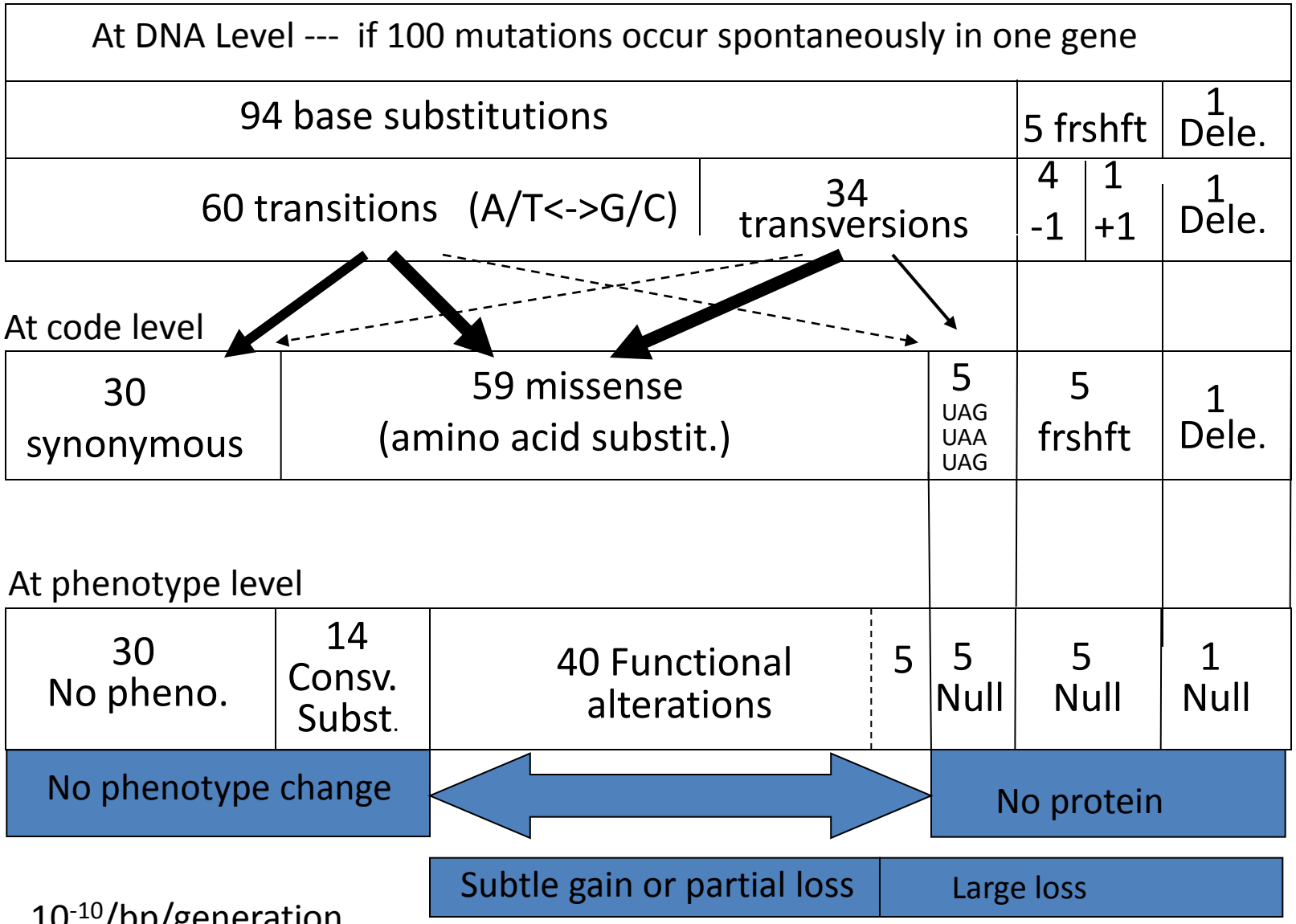
Using whole genome sequencing to identify duplications (inversions and deletions) by their novel sequence join points



Using genome sequencing technologies to identify and define duplications and amplifications. A. Uniform coverage of a genome in which region 4-7 is duplicated. B. After assembly of the sequence it is apparent that there is double coverage for the region 4-7 relative to the remainder of the genome. Coverage will be increased in proportion to the copy number of amplification. C. Identification of unique junction sequence, 7/4, not found in the reference sequence, specifies precisely the length of the region duplicated.

Sun et al 2013 PLoS One

Distribution of spontaneous mutations APPEARING in ONE GENE



10^{-10}/bp/generation
Point mutation rate

See Patricia Foster: Proc Natl Acad Sci U S A. 2012

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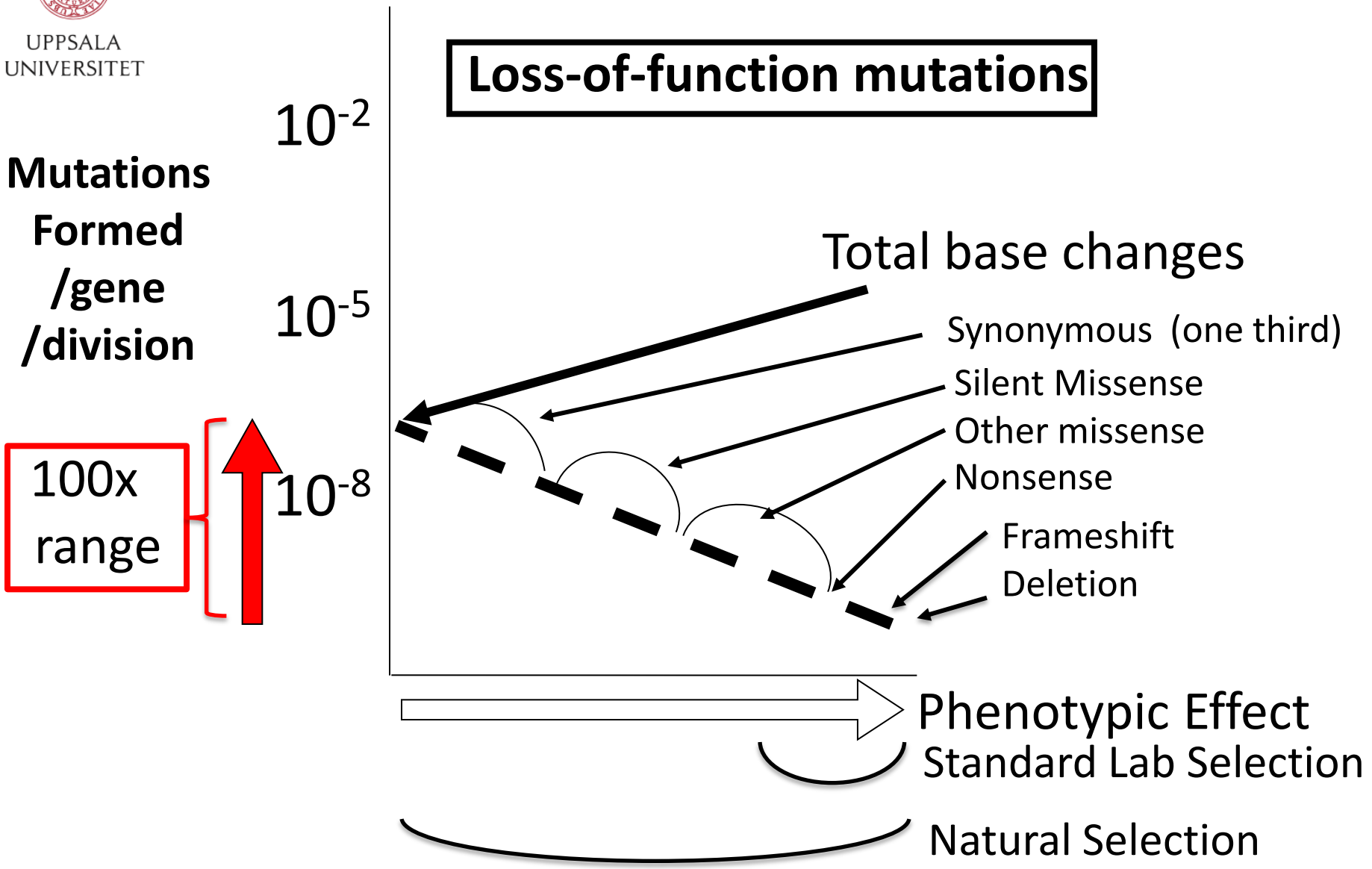

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Gain-of-function mutations