Chapter 4: Strategies for studying inactivation mechanisms

1. Common experimental approaches

1.1. Relationship between inactivation and functional or morphological alterations

Microbial inactivation results in a loss of the ability to reproduce under favorable conditions. However, a cell can become non-culturable without being immediately lysed or morphologically altered. The methodological approach therefore consists of **correlating** the kinetics of viability loss with the appearance of measurable damage.

a) Functional alterations (metabolic and physiological)

Type of alteration	Measurement method	Interpretation
Membrane permeability	Entry of markers (propidium iodide, SYTOX Green)	Loss of membrane barrier integrity
Membrane potential	Fluorochrome ($DiOC_3(5)$, JC-1)	Dysfunction of respiratory chains
Intracellular enzyme activity	Tetrazolium salt reduction test (XTT, MTT, CTC)	Loss of metabolic activity
Protein synthesis	Incorporation of labeled methionine (^{35}S-Met)	Translation arrest
DNA integrity	Pulsed-field gel electrophoresis (PFGE), Comet assay	DNA fragmentation or damage

Example:

For a heat treatment at 60 °C on *E. coli*:

- A 5-log loss of viability is observed after 5 minutes.
- Membrane permeability (IP entry) appears only after 10 minutes. → Death occurs before loss of membrane integrity, suggesting another primary target (e.g., denaturation of key enzymes).

b) Morphological alterations

Technique	Information obtained
Transmission electron microscopy (TEM)	State of membrane, nucleoid, presence of vacuoles, lysis
Scanning electron microscopy (SEM)	Surface modifications (blebs, perforations, roughness)
Fluorescence microscopy (with specific markers)	Visualization of DNA (DAPI, Hoechst), membrane (FM4-64), ROS (DCFH-DA)

1.2. Use of specific mutants

Mutants are powerful tools for **demonstrating the role** of a target or a repair pathway in inactivation.

✓ **Methodological principles**

- A **wild-type** (WT) strain and a **mutant** strain deficient for a given function are compared.
- If the mutant is **more sensitive** → the missing function is protective.
- If the mutant is **more resistant** → the missing function contributes to sensitivity (e.g., activation of a toxin).

✓ **Examples of commonly used mutants**

Gene / function	Expected phenotype	Interpretation
***recA* (DNA repair by recombination)**	Hypersensitive to UV and ionizing radiation	DNA is a major target
***polA* (DNA polymerase I)**	Hypersensitive to alkylating agents	Role in single-strand break repair
***mutS* (mismatch repair)**	Moderate sensitivity to certain agents	Confirms the role of DNA lesions
***soxS* / *oxyR* (oxidative stress response)**	Hypersensitive to H_2O_2	Oxidative stress is involved

***ΔclpP* (protease degrading damaged proteins)**	Sensitivity to heat	Denatured proteins are critical
***ΔmscL* (mechanosensitive channel)**	Sensitivity to osmotic shock	Internal pressure regulation is important

To confirm that the phenotype is indeed due to the mutation, **complementation** is performed: the wild-type gene is reintroduced on a plasmid. Return to normal sensitivity validates the role of the gene.

2. Cellular targets involved in inactivation by different agents

Each inactivating agent has one or more **primary targets** (those whose damage triggers death) and **secondary targets** (aggravating the damage). The table below summarizes current knowledge.

2.1. Detailed table of targets by agent

Inactivating agent	Primary target (major)	Secondary target(s)	Main molecular mechanism
Moist heat (60-100 °C)	Proteins (denaturation)	Membranes, RNA, repair enzymes	Loss of tertiary structure, protein aggregates
Dry heat (160-180 °C)	DNA (breaks)	Proteins, membranes	Base oxidation, depurination
UV-C (254 nm)	DNA	Ribosomes (secondary effect)	Pyrimidine dimers (cyclobutane and 6-4 PP)
Ionizing radiation (γ, e^-)	DNA (double-strand breaks)	Membrane lipids (peroxidation)	Free radicals (•OH), single/double-strand breaks
Hydrogen peroxide (H_2O_2)	DNA (base oxidation)	Fe-S center proteins, membrane	Oxidative damage, single-strand break

Chlorine / hypochlorite (NaClO)	Membrane (oxidation)	Proteins, DNA (weak)	Fatty acid oxidation, disulfide bridges
Ozone (O_3)	Membrane (peroxidation)	DNA, proteins	Cell wall rupture, radical formation
Ethanol / alcohols	Membrane (fluidization)	Proteins (partial denaturation)	Metabolite leakage, transport inhibition
High hydrostatic pressure (HHP)	Ribosomes (dissociation)	Membrane, proteins	Dissociation of ribosomal subunits
Antibiotics (by class)	Variable (cell wall, DNA gyrase, ribosome, etc.)	Energy metabolism	Specific inhibition of a molecular target

2.2. Methodological approach to identify the primary target

A rational strategy combines several approaches:

1. **Comparative kinetics**: The primary target is damaged before or at the same time as loss of viability (not after).
2. **Use of mutants**: A mutant hypersensitive to the agent indicates that the missing pathway protects against damage to a specific target.
3. **Direct biochemical tests**:
    - DNA: extraction and analysis by electrophoresis, long-range PCR, HPLC-MS for modified bases.
    - Proteins: electrophoresis (aggregates), residual enzyme activity.
    - Lipids: gas chromatography (peroxidation).
4. ***In situ* fluorescent labeling**:
    - TUNEL (fragmented DNA)
    - Annexin V (phosphatidylserine exposure → membrane alteration)
    - DHE probe (detection of superoxide anion)

2.3. Notion of sublethal cells

A major methodological difficulty is the existence of **damaged but not inactivated** cells (viable but altered). They are particularly important in food and medical industries because they can **repair their damage** after treatment.

Consequences for studying mechanisms:

- Distinguish between immediate vs. delayed inactivation (culture on non-selective medium + possible reactivation).
- Sublethal cells may show alterations without loss of cultivability → false identification of primary target if viability is not tracked over time.

Methodological recommendation: Always couple viability measurement (plate counting) with alteration measurement (e.g., flow cytometry) at multiple times after treatment (0 h, 1 h, 3 h, 24 h).

3. Decision tree for studying a new inactivating agent

1. **Step 1**: Determine the mortality kinetics (survival curve).
2. **Step 2**: Observe morphology (electron or fluorescence microscopy).
3. **Step 3**: Test known mutants (DNA repair, oxidative stress response, membrane integrity).
4. **Step 4**: Measure specific damage (DNA, proteins, lipids) at sublethal and lethal doses.
5. **Step 5**: Compare with a reference agent whose target is known (e.g., UV for DNA, heat for proteins).
6. **Step 6**: Propose a mechanistic model (e.g., "Agent X inactivates via oxidative DNA damage with a membrane contribution").