Control of Bacterial Growth

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Introduction

- Control of growth of microorganisms is essential to prevent contamination, stop the spread of disease and infection, and reduce food spoilage.
- Using physical or chemical agents allows for control of microbial growth.
- Physical control is achieved by heat, radiation or filtration.
- Chemical control is achieved by many different natural and synthetic agents; including antibiotics, bleach, ethanol, hydrogen peroxide and more.
- Different bacteria can require different methods of control depending on cell wall structure, presence of a spore, location, and more.

Terminology

- **Sterilization**: to kill or remove all microorganisms
- **Autoclave**: device that pressurizes to create heat and steam for sterilization.
- **Thermal Death Time**: time needed to kill all cells at a specific temperature.
- **Thermal Death Point**: minimum temperature at which all cells are killed within 10 minutes.
- **Pasteurization**: using heat to reduce the number of cells in a liquid.
- **Bactericidal**: agents that kill bacteria.
- **Bacteriostatic**: agents that inhibit growth of bacteria.
- **Minimum inhibitory concentration**: minimum concentration of antimicrobial agent needed to inhibit the growth of an organism.
- **Sterilant**: an agent that kills all microorganisms including spores.
- **Disinfectant**: an agent that kills microorganisms, but not spores. (Used on Surfaces)
- **Sanitizer**: an agent that reduces the number of microorganisms. (Used on food surfaces)
- **Antiseptic**: an agent that kills microorganisms or inhibits their growth, but is non-toxic to animals. (Used on living tissue)

- Madigan *et. al.*, 2018, pp.164-170

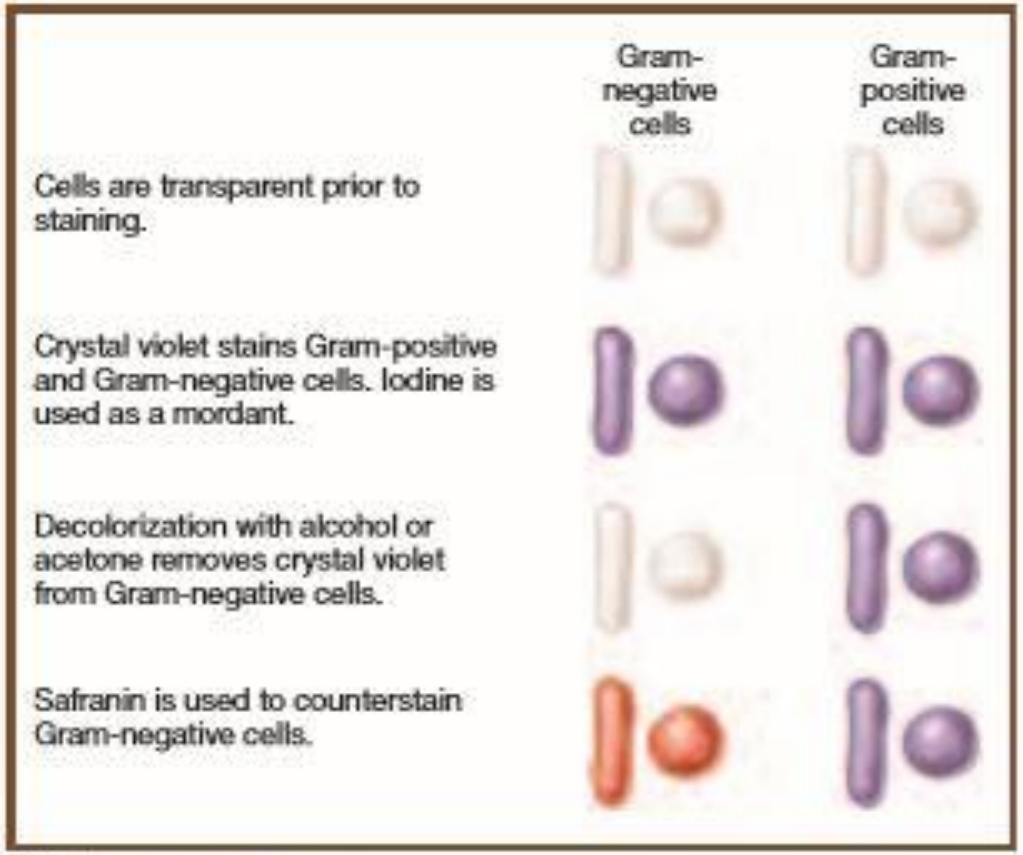
Differential Staining

- Gram Staining (cell wall structure)
  - Differentiates cell wall structure
- Endospore Staining (spore production)
  - Steam is needed to force the primary stain into the spore
- Capsule Staining (capsule production)
  - Extracellular capsule made up of polysaccharides or polypeptides is resistant to stains

Gram Staining

Use 24 hr culture

- Crystal violet (1 min)
- rinse with water
- iodine as mordent (1 min)
- rinse with water
- decolourize (~20 sec)
- rinse with water
- counterstain with safranin (~50 sec)
- rinse with water and blot dry
- **gram positive cells keep purple crystal violet and gram negative cells are reddish-pink with safranin**



6-1 GRAM STAIN After application of the primary stain (crystal violet), decolorization, and counterstaining with safranin, Gram-positive cells stain violet and Gram-negative cells stain pink/red. Notice that crystal violet and safranin are both basic stains, and that it is the decolorization step that makes the Gram stain differential.

Leboffe & Pierce, 2011, p. 45

Staining Tips

- Make slides first and allow to air dry while you perform other tasks
- Easier to use plate culture on a drop of water; do not use a drop of water if you take a loop of broth culture instead
- Spread the culture thin to air dry quickly
- Record colour(s) seen, shape and arrangement of cells and measure cell length

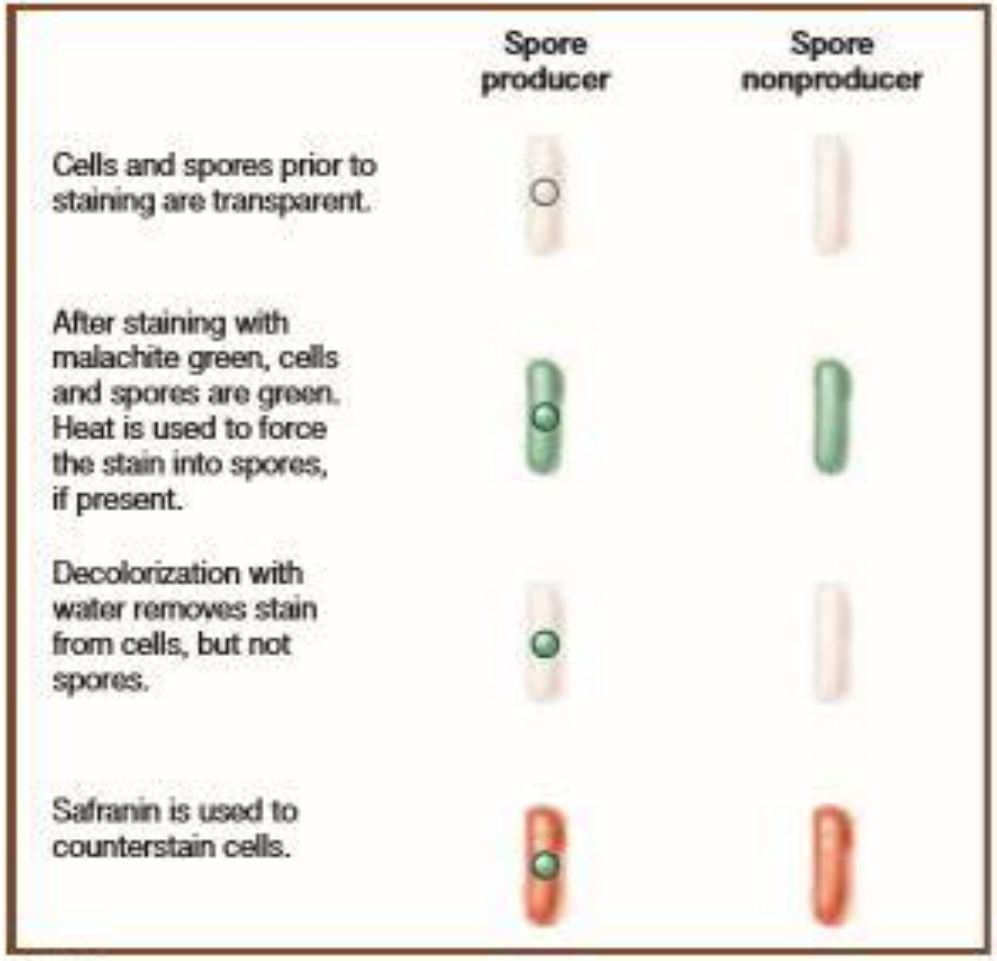
- **Always work over a stain tray**
- **Liquid waste is poured into chemical waste jug in fume hood**
- **Paper square in solid waste beaker with orange biohazard bag**

Endospore Staining

Use 48hr culture

- Malachite green steamed into spore, kept moist with a paper towel square and add stain to prevent it from drying (fume hood) for 5 min
- rinse with water
- counter-stain with safranin (60-90 seconds)
- rinse with water and blot dry

- **spores are green and vegetative portion of cell is pinky-red**

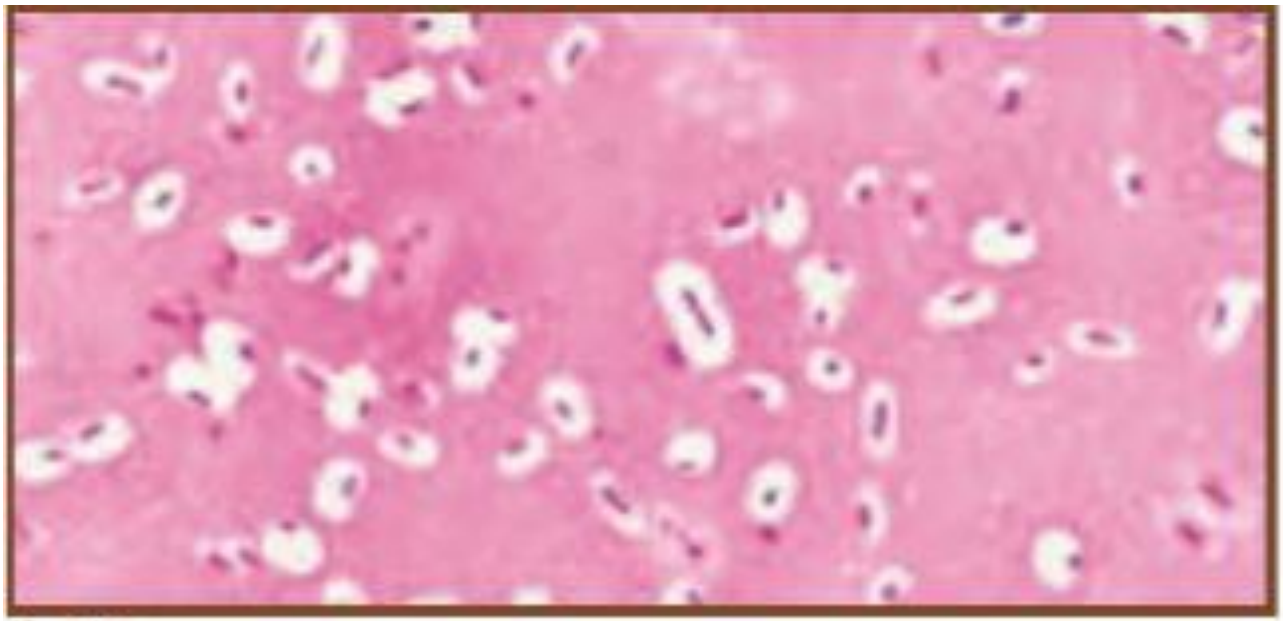


6-22 The Schaeffer-Fulton Spore Stain Upon completion, spores are green, and vegetative and spore mother cells are red.

Leboffe & Pierce, 2011, p. 52

Capsule Stain

- Acid stain and basic stain to colour within cells and the background
- **capsules show as clear halos around cells**



6-20 CAPSULE STAIN OF *KLEBSIELLA PNEUMONIAE* The acidic stain colorizes the background while the basic stain colortzes the cell, leaving the capsules as unstained, and white clearings around the cells. Notice the lack of uniform capsule size, and even the absence of a capsule in some cells. Compare this micrograph to Figure 6-21. The difference in cell size between the two photos is due to enlargement of the micrograph, not to the staining.

Leboffe & Pierce, 2011, p. 51

Antibiotics Terminology

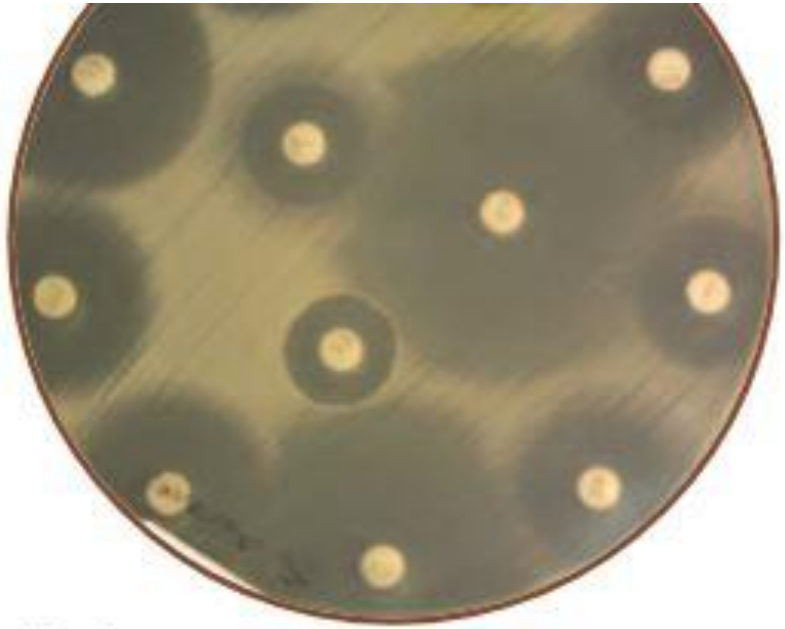
- **Selective toxicity**: inhibits growth or kills pathogens without affecting the host
- **Narrow spectrum**: kills or inhibits growth of select bacteria
- **Broad spectrum**: kills or inhibits growth of many bacterial types
- **Antibiotic class**: grouping of chemical structure of the antibiotic
- **Mode of action**: mechanism(s) that the antibiotic targets on or in the bacterial cell

- Madigan *et. al.*, 2018, pp. 852-858

Kirby-Bauer Test
Antibiotic Susceptibility by Disk Diffusion

- A swab is used to coat the whole surface of the agar plate with the pure culture of the bacteria
- Antibiotic disks are placed on the surface of the bacterial lawn.
- The antibiotic will diffuse into the agar with the lowest concentration being the farthest from the disk.
- The zone of inhibition around the disk is due to the lack of growth of bacteria in that area inhibited by the antibiotic.
- The diameter of the zone is measured and compared to known standards to interpret if the bacteria is sensitive or resistant to the antibiotic.

- Madigan *et. al.*, 2018, p. 839



19-1 Disk Diffusion Test of Methicillin-Susceptible *Staphylococcus aureus* This plate illustrates the effect of (clockwise from top outer right) Nitrofurantoin (F/M300), Norfloxacin (NOR 10), Oxacillin (OX 1), Sulfisoxazole (G 25), Ticarcillin (TIC 75), Trimethoprim-Sulfamethoxazole (SXT), Tetracycline (TE 30), Ceflizoxime (ZOX 30), Ciprofloxacin (CIP 5), and (inner circle from right) Penicillin (P 10), Vancomycin (VA 30), and Trimethoprim (TMP 5) on Methicillin-resistant *S. aureus*. Compare the zone sizes with those in Figure 19-2, paying particular attention to Ceflizoxime, Oxacillin, and Penicillin.

Leboffe & Pierce, 2011, p. 222

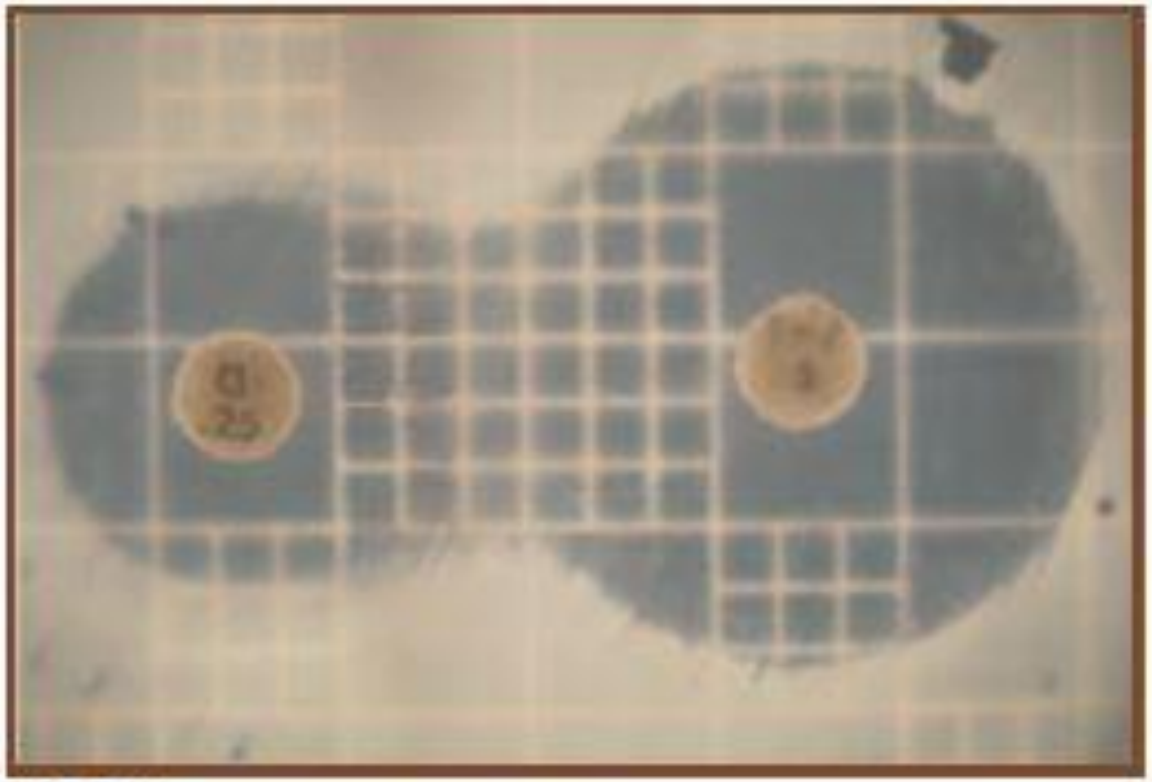
Kirby-Bauer Procedure

Antibiotic Disk Susceptibility

- Label two 100mm Mueller-Hinton plates with group #, date, MH, organism (*E. coli* or *B. subtilis*)
- Use 24 hr culture broth
- Use only ONE cotton swab to spread 24hr culture on two 100mm MH plates
- Completely cover plates with culture and let sit with lid on for 2-3 minutes
- Use tweezers (flamed and cooled) to place 3 different discs on each swabbed plate
- Leave upright for a couple minutes, then parafilm
- Measure diameter (mm) of clearing compare to Table 2.2 to indicate if bacteria are resistant, susceptible or intermediate

Combined Effects of Antibiotics

- Combining two antibiotics that have a similar mode of action can increase the effect that each would have individually.
- **Synergistic** effect is when both antibiotic disks show a combined zones of inhibition on the swabbed agar plate.
- **Additive** effect is when both antibiotic disks do not have a combined effect. Two distinct zones of inhibition on the swabbed agar plate.
- **Antagonistic** effect is when one antibiotic interferes with the action of the other antibiotic. Smaller zones of inhibition than usual.



19-5 ANTIBIOTIC SYNERGISM This is an example of synergism between the antibiotics Sulfisoxazole (G) and Trimethoprim (TMP). The numbers on the discs represent micrograms (μg) of antibiotic.

Leboffe & Pierce, 2011, p. 225

Combined Effects Procedure

- Label two 100mm MH plates with group #, date, organism, MH, antibiotics used
- On bottom place a small dot in the very centre of the plate with your sharpie, then measure and ‘dot’ or ‘x’ the distances indicated for each combination of antibiotics (Tri-Tet 14mm apart, Tri-Sulf 12.5mm apart)
- Use the 24 hr broth culture
- Use only ONE swab to spread culture over complete surface of both MH plates, let dry for a couple minutes
- Use tweezers (flamed and cooled) to place the two antibiotic discs on the measured places, let sit for a couple minutes
- Parafilm the plates and then use coloured scientific tape from your drawer to tape together all 4 MH plates with antibiotic discs
- Record if clearing is combined or separate

Minimum Inhibitory Concentration Commercial test strips

- Different companies produce strips of a gradual gradient of antibiotic concentration for testing the minimum inhibitory concentration (MIC)of an antibiotic on an organism.
- E Test is short for Epsilon test
- E test strips (bioMérieux)
- M.I.C.E. strips (Oxoid)

19-6 E-TEST The E-test is a procedure in which susceptibility to a particular antibiotic can be quantified as a Minimum Inhibitory Concentration (MIC). After incubation, the MIC is determined by where the zone of inhibition intersects the scale printed on the strip. **A** Shown is the zone formed by the antibiotic vancomycin when incubated with Methicillin-resistant *Staphylococcus aureus* (MRSA). **B** The antibiotic penicillin is generally not effective against Gram-negative bacteria. Shown is *Escherichia coli* grown with a Penicillin G strip. Note the absence of an inhibition zone.

Leboffe & Pierce, 2011, p. 225

Minimum Inhibitory Concentration
E Test Strip Procedure

- Use swab to spread culture over complete surface of the MH plate, let dry for a couple minutes
- Use tweezers (flamed and cooled) to place the E Test strip on the centre of the plate, let sit for a couple minutes
- Read and record the results (record the antibiotic name also)
- Each strip contains one type of antibiotic at a gradient of concentrations (0.016 μg/μl to 256 μg/μl)
- Where clearing begins is the lowest concentration required to inhibit growth; look for colonies growing within the clearing (mutants)

Bleach Effects

- Household bleach is a solution of ~5-7% sodium hypochlorite used as a common disinfectant and sanitizer.
- The chlorine compound acts as an oxidizing agent that causes the unfolding of essential bacterial proteins, which then causes the proteins to clump and become non-functional (Winter *et.al.*, 2008)

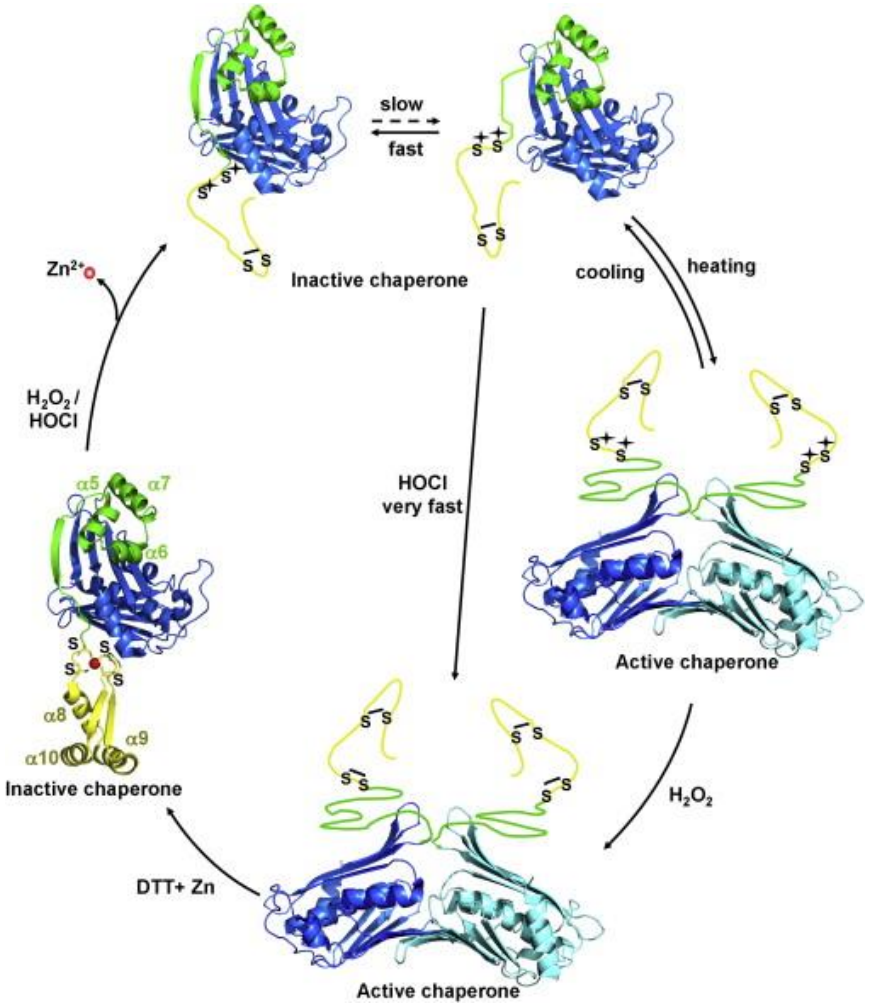


Figure 7. Hsp33—A Paradigm for HOCl's Oxidative Unfolding Activity (Winter *et.al.*, 2008)

Bleach Effects Procedure

- Label the 60mm tryptic soy agar plate (TSA) with your group #, date, organism, age of bleach
- Use 24 hr broth culture
- Use only ONE cotton swab to spread culture over whole surface of the plate and let sit with lid on for a couple minutes
- With tweezers (flamed and cooled), dip clean filter paper disk into bleach solution, touch to side of tube to remove excess, then place in centre of plate for 10 minutes (lid on while you wait)
- Remove used disc into orange biohazard bag and parafilm plate. Place plate at the side bench with all completed bleach plates
- Measure diameter of clearing in mm and describe amount of growth on rest of plate for all 10 plates

Temperature: Lethal Effects

- High temperatures cause proteins to denature (unfold).
- Mesophiles survive in temperature ranges between ~10^{o}C and ~45^{o}C
- Some mesophilic bacteria can survive temporarily outside of their cardinal temperature range by producing spores.
- By testing bacterial survivability at various temperatures, we can identify the Thermal Death Point and confirm the ability of a bacteria to produce spores.

- Madigan *et. al*., 2018, pp. 152-153

Temperature Effects Procedure

- Label a 100mm TSA plate with group #, date, organism; draw in two lines across the diameter to make four sections and label with temperatures (40, 60, 80, 100$_{o}$C)
- Use 48 hr broth culture tube labeled with ‘temp’, add your coloured scientific tape with group # onto the glass of the tube
- **Taking your tube in the test tube rack and using tongs**, place the tube into the 40$_{o}$c water bath for 10 min, remove using tongs and take back to your bench
- With your loop, aseptically, simple streak in the 40$_{o}$C section of your 100mm TSA plate
- Place tube into the 60$_{o}$C bath for 10 min, repeat as above, then repeat for 80$_{o}$C and 100$_{o}$C (**caution hot water produces steam and can burn, must use tongs and heat resistant gloves provided by bath**)
- Parafilm the plate, incubate for 24 to 48 hrs and then record amount of growth
- Growth is recorded as **+++** = a lot, **++** = moderate, **+** = low, **-** = no growth

References

- Leboffe MJ and Pierce BE (2011) *A Photographic Atlas for the Microbiology Laboratory*, 4th edition. Morton Publishing Company, Colorado.
- Madigan MT, Bender KS, Buckley DH, Sattley WM, and Stahl DA (2018) *Brock Biology of Microorganisms*, 15th edition. Pearson Education, Inc., New York.
- Winter J, Ilbert M, Graf PCF, Ozcelik D, and Jakob U (2008) *Bleach Activates a Redox-Regulated Chaperone by Oxidative Protein Unfolding*. Cell 135 (4), pp. 691-701.