

Experiment	Preparation and Isolation of <i>lac</i>⁻ mutants
Advisor	Romana Maibach; maibach@immv.unizh.ch
Reading	- Chapters in BBOM 9th: 9.1; 9.3; 9.4, 9.5, 9.8, 5.4, 7.2, fig. 10.5 BBOM 9th: Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, (BBOM, International Edition), Prentice Hall, 1999. ISBN: 0-13-085264-3. - Chapter 15.5 in Lengeler J.W., G.Drews, H.G.Schlegel (eds): "Biology of the Prokaryotes", Thieme Stuttgart, 1999. ISBN: 3-13-108411-1. - Chapters 16.1.3; 15.1.5 in Schlegel G. H.: "Allgemeine Mikrobiologie", 7. Auflage, Thieme Stuttgart, 1992. ISBN: 3-13-444607-3.
Objectives	- Making and detection of mutants with defects in the lactose operon phenotypically distinguishing <i>lacZ</i> from <i>lacY</i> mutants Isolation of <i>lac</i>⁻ mutants and calculation of their relative frequency
Background	In this exercise, you will obtain lactose negative mutants of <i>E.coli</i> and classify them as either <i>lacZ</i> or <i>lacY</i>. Among other things, lactose metabolism requires two specific proteins: - A permease which transports lactose into the cell. The permease is the product of the <i>lacY</i> gene. β-galactosidase, an enzyme that hydrolyzes lactose to yield glucose and galactose. β-galactosidase is the product of the <i>lacZ</i> gene. A cell which fails to synthesize either of these proteins, or in which one of these proteins is defective, will be unable to use lactose. Thus, if either the <i>lacZ</i> gene or the <i>lacY</i> gene is damaged so that it codes for a defective protein, that cell and all of its progeny will be unable to use lactose. The shorthand notation for this is <i>lac</i>⁻. If we want to specify the defective genes we say <i>lacZ</i> or <i>lacY</i>. Such mutants may occur spontaneously in a population, but are very rare; on the order of 10⁻⁸; or one <i>lac</i>⁻ cell in 100 million. We can distinguish <i>lac</i>⁺ from <i>lac</i>⁻ colonies on an indicator medium. We shouldn't have more than 200 well separated colonies on a plate; thus to detect a spontaneous <i>lac</i>⁻ colony, we would have to screen about half a million plates. With certain chemicals, called mutagens, the mutation rate increases greatly (10⁻⁴ to 10⁻⁵ instead of 10⁻⁸). In this experiment, ethyl methane sulfonate (EMS) is used as the mutagen. Even with the mutagen, we would have to screen 100 to 1000 plates to isolate one mutant! We will, therefore, use the technique of ampicillin selection to further enrich for <i>lac</i>⁻ mutants. Ampicillin selection technique: Ampicillin (an antibiotic of the penicillin family) kills only growing cells. Selection depends on the fact that <i>lac</i>⁻ cells don't grow in minimal medium if lactose is the only carbon- and energy-source. Thus, if we suspend a mutagenized culture in lactose minimal medium and add ampicillin, most of the <i>lac</i>⁺ cells will be killed, while the non-growing <i>lac</i>⁻ cells will survive. After this treatment, we wash away the ampicillin and resuspend the remaining cells in broth to allow all cells to grow. If 99% of the <i>lac</i>⁺ cells died as a consequence of the ampicillin treatment, we will have achieved a 100-fold enrichment of <i>lac</i>⁻ cells. The procedure can be repeated to further enrich, but remember that ampicillin-resistant <i>lac</i>⁺ cells will also survive. After more than two rounds of selection, the enrichment success tends to decrease due to the increased numbers of ampicillin-resistant cells. - Ordinary <i>E. coli</i> culture: <i>lac</i>⁻ frequency about 10⁻⁸ - EMS-treatment: increases <i>lac</i>⁻ frequency to about 10⁻⁵ - Two times ampicillin selection: <i>lac</i>⁻ frequency about 10⁻¹

You will get an *E. coli* culture (EMS 229) which has already been treated with EMS and twice selected with ampicillin.

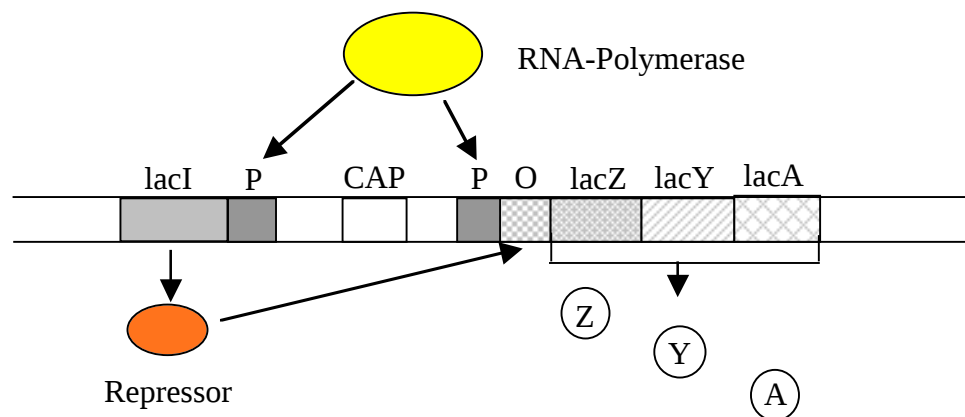
MacConkey-Lactose medium is a carbohydrate fermentation indicator medium. *Lac*⁺ colonies are red (formation of acid), while *lac*⁻ colonies are white.

IPTG (isopropylthiogalactoside) is a special chemical called an **inducer**. *E. coli* cells normally express the *lacZ* and *lacY* genes only in the presence of lactose. IPTG is chemically just different enough from lactose that it cannot be metabolized by the *E. coli* cell, but it can induce the *lacZ* gene and allow β -galactosidase to be made.

If a cell or colony is *lacZ*⁻, it cannot make active **β -galactosidase** under any circumstance, and lactose will not be hydrolyzed (*lacZ*⁻ colonies are white whether or not IPTG is present in MacConkey-Lactose medium).

If a cell is *lacY*⁻, it cannot transport lactose. When IPTG is added β -galactosidase will be made. The concentration of lactose in MacConkey plates is high enough so that some lactose diffuses into the cell and is hydrolyzed by the β -galactosidase (*lacY*⁻ colonies will be **white** on ordinary MacConkey-Lactose medium, but **red** on the MacConkey-Lactose medium with IPTG in it).

Regulation of the *lac* –Operon:



- **Without lactose:** repressor binds to the operator (O) and represses transcription by RNA-polymerase.
- **With lactose:** lactose binds to the repressor. The lactose-repressor complex is unable to bind to the operator (O). Transcription takes place.

Literature

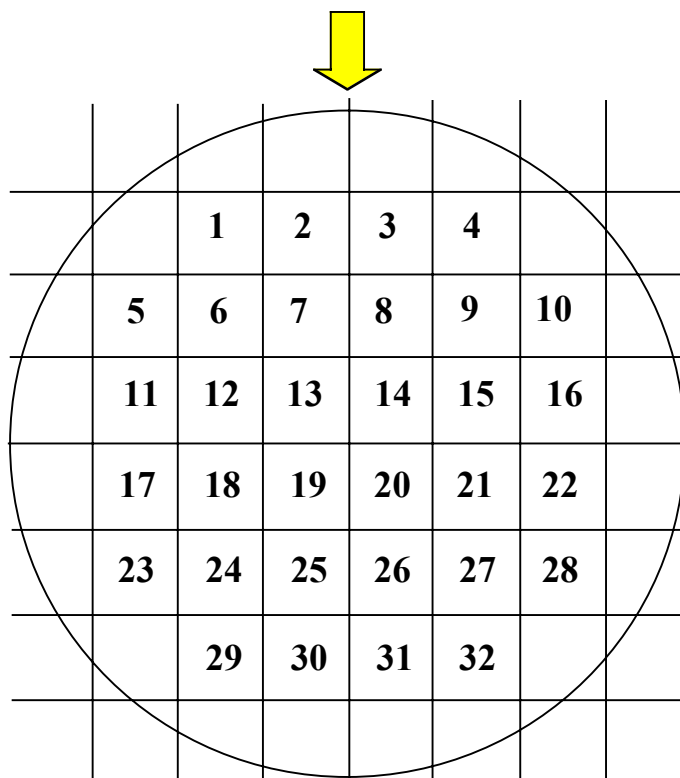
www. Links

<http://medic.med.uth.tmc.edu/path/macconk.htm>
<http://esg-www.mit.edu:8001/esgbio/pge/lac.html>

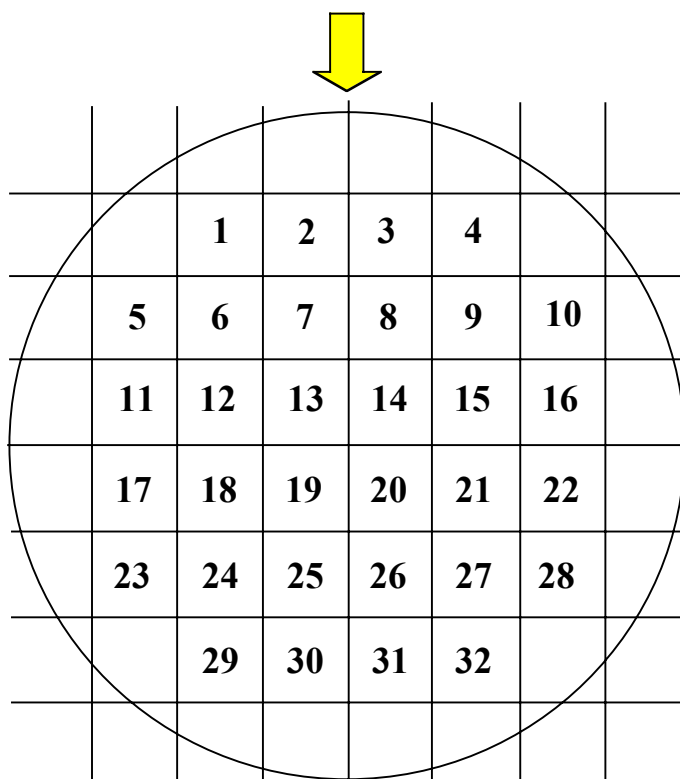
Practical Work	1. Pipette 0.1 ml of the EMS 229 liquid culture onto a MacConkey-Lactose plate. 2. Spread it over the surface using a glass spreader (Drigalski-spreader). 3. Your plate will be incubated for 24 to 48 hours at 32°C. (For the second part of the experiment, you will get the incubated plates of the group that performed the experiment the day before) 4. Examine the plate for white colonies (<i>lac</i>⁻ mutants). 5. Count the total number of red and white colonies. 6. You will get one ordinary MacConkey-Lactose plate and one plate with MacConkey-Lactose + IPTG medium (marked "I"). Put each plate on the plate matrix diagram with the mark you drew lined up next to the arrow. 7. Take a sterile toothpick out of the petri dish using the forceps and hold it at one end (only touch one end!). Touch the sterile end of the toothpick to one of the white colonies you obtained, then touch it to the space marked "1" on the MacConkey-Lactose agar and next to the space marked "1" on the IPTG agar. Repeat this procedure for each of (up to) 32 white colonies. Be sure to use a fresh sterile toothpick each time! 8. Incubate your plates at 32°C. Return after 24 hours to examine your plates.
Materials	<i>E. coli</i>, one glass spreader, 1 ml pipettes, 35 toothpicks, 3 petri dishes, alcohol for flaming Chemicals: IPTG, MacConkey-Lactose agar
Laboratory Rules & Precautions	Material that was in touch with mutagenized organisms must be disposed off by sterilization. It is necessary to work cautiously and aseptically. Use good laboratory practice! Do not contaminate yourself, others or the laboratory environment. All waste must be sterilized before disposal. Please wash your hands before you leave the room and disinfect bench surfaces with 70 % ethanol.
Goals & Experiences gained	• Experience how a bacterial culture can be mutagenized • Understand how desired mutants can be selected • The function and regulation of lactose metabolism
Timing	Ca. 1 hour on the first day and 15 min on the second day
Reporting	Report the following: • Total white colonies • Total red colonies • Frequency of <i>lac</i>⁻ colonies = $\#white / (\#white + \#red) =$ • Number of white colonies "picked": • Number of white colonies on MacConkey-Lactose: • Number of white colonies on MacConkey-Lactose + IPTG: • Number of red colonies on MacConkey-Lactose + IPTG: • Number of colonies which were <i>lacZ</i>: • Number of colonies which were <i>lacY</i>: • Proportion of <i>lacZ</i> / <i>lacY</i> :
Questions to be answered	What other mutagens do you know ? How do they induce mutations? Can you explain the concept of the Ames Test?

Appendix

Plate matrix diagram: Put each plate on the matrix below with the mark at the arrow.



Lactose + IPTG



Lactose