UNIVERSITY OF COPENHAGEN

Microbiology 2010

Block 3: manual of laboratory exercises

Jørgensen, Niels O. G.; Sørensen, Jan; Nybroe, Ole

Publication date:
2010

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Jørgensen, N. O. G., Sørensen, J., & Nybroe, O. (2010). *Microbiology 2010: Block 3: manual of laboratory exercises*. Department of Agriculture and Ecology, University of Copenhagen.

UNIVERSITY OF COPENHAGEN

Microbiology 2010

Block 3
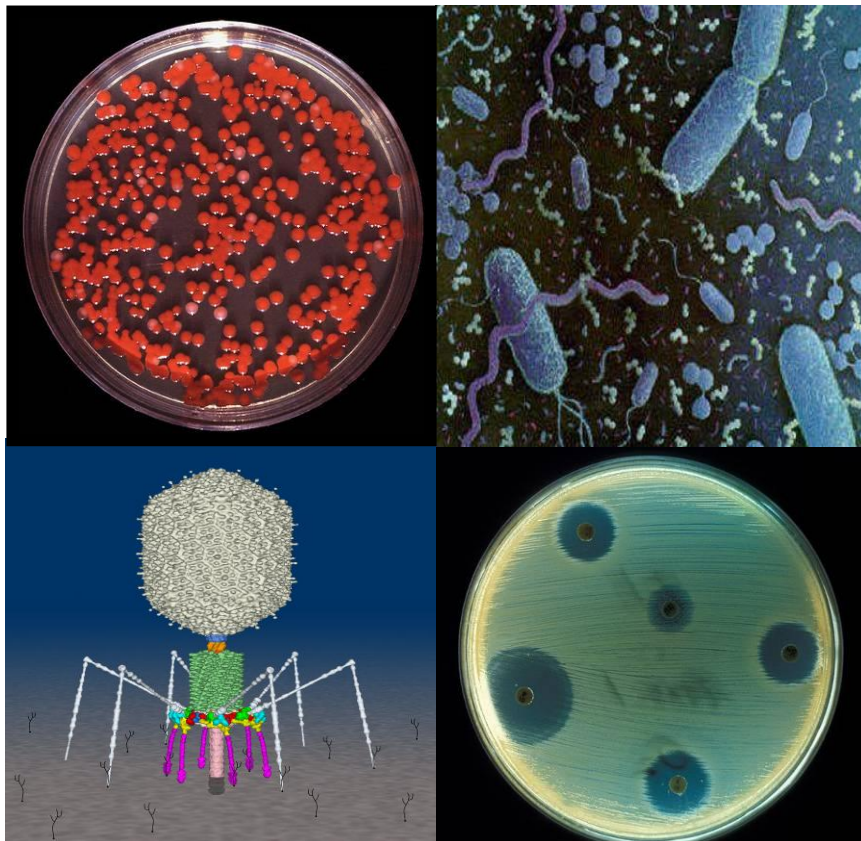
Manual of laboratory exercises

Section of Genetics and Microbiology

Department of Agriculture and Ecology

University of Copenhagen

List of exercises in Microbiology

No.	Title of exercise	Page	Week								Report
			1	2	3	4	5	6	7	8	
1	Bacterial cultures and cultivation of bacteria	1	■								No
2	Microscopy of bacterial cells	4	■	■							No
3	Characterization of bacterial cultures	7	■	■	■						No
4	Identification of bacterial cultures	11		■	■						No
5	Growth of bacteria	16				■	■				Yes
6	Environmental effects on growth of bacteria	23				■	■				Yes
7	Inhibition of bacterial growth by UV radiation	28				■	■				Yes
8	Bacteriophages	32						■	■		Yes
9	Bacterial conjugation	38						■	■		Yes
10	Selection of bacterial mutants	42						■	■		Yes
11	Microbial metabolism in soil (NR only)[*]								■	■	Yes
12	Nitrification in soil (NR only)[*]								■	■	Yes
13	BOD as measure of organic matter degradation (NR only)[*]								■	■	Yes
	Cultures, media and reagents	47									

[*]Manual of Exercise 11, 12 and 13 for NR students will be available on Absalon one week before start of the exercises

COMMENTS

Time and location

Times of the exercises are Tuesday at 10:00 – 12:30 and Thursday at 10:00 – 12.30
The laboratories are located on Thorvaldsensvej 40, entrance 2, 2nd floor, rooms J217 and F217

Duration of exercises

Most exercises last two weeks. In the first week, bacterial cultures are transferred to specific media, diluted or otherwise treated. In the following week, the grown-up bacteria are characterized, e.g., by microscopy or counting of colonies. This two-week schedule means that you cannot expect to complete an exercise in just one week.

Reports

No written reports are produced from Exercise 1 to 4. The obtained results are discussed with the teachers.

Written reports are obligatory for exercise 5 to 10. Also, NR students must deliver reports for exercise 11, 12 and 13. The reports must be handed in the week after completion of the exercise. Results from the exercises are presented by the students as part of the group work presentations. The teachers will comment the reports within two after they are handed in.

Attendance

The students must attend at least 75 % of the exercises to pass the laboratory course.

More information on teaching schedules and course curriculum for Biotech and NR students can be found at the Absalon website.

EXERCISE 1: BACTERIAL CULTURES AND CULTIVATION OF BACTERIA

Introduction

Work with bacteria in pure culture - aseptic technique
Since many of the laboratory exercises will be made with a pure culture – that is, a single strain (or clone) of a bacterial species – we must be able to maintain it in a sterile condition, free of other strains. Inoculation of a sterile medium with a pure bacterial culture without outside contamination is referred to as **aseptic** technique. To master aseptic technique is fundamental for work in a microbiological laboratory.

Purpose of the exercise
To practice the basic skills necessary for aseptic technique, including inoculation of bacterial cultures in liquid and on solid media.

Materials per team
Basic equipment (flame, needle)
1 Glass tube with cap
1 Petri plate with high-agar medium

Team size
1 student per team

Practical work

All basic training in this exercise will be performed using water inoculum (without bacteria) and pure high-agar plates (without growth medium). All steps (marked by insert and dark points) represent the practical work and must be performed. The procedures of the exercise will be further practiced in Exercises 2 and 3.

Inoculation of liquid media (in tubes or flasks)
An amount of bacterial cells – the inoculum – is transferred (inoculated) into the sterile medium using a special needle and with special precautions.

- The needle (or loop) should be heated by flaming immediately before and after making the transfer. Hold the needle downwards and into the flame, to heat both the needle and lower part of the handle. **Check Figure 1**.

- During transfer, hold the tube in the left hand and hold the plug or cap between the fingers of the right hand. Caution: Never lay a plug or cap down! Hold the tube as nearly horizontal as feasible during transfer and do not leave it open longer than necessary. The mouths of the tube from which culture is taken should also be passed through the flame immediately before and after the needle is introduced and removed. Same procedure for the tube into which the cultures are transferred. **Check Figure 2**.
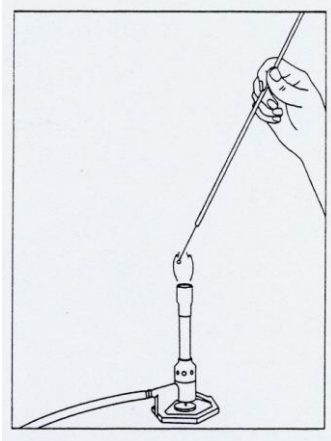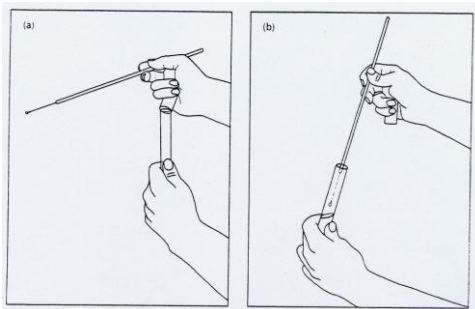
Figure 1

Figure 2

Inoculation of solid media (agar plates)
A transfer of inoculum using the needle is called streaking – a Petri plate so prepared is a streak plate.

- Transfer a drop of culture at one edge of the agar using the sterile needle. Flame the streaking needle and cool it by jabbing it into another edge of the agar.

- With the drop of bacteria away from you, streak the culture back and forth from edge to edge in 2 or 3 parallel lines, moving toward you. Be careful not to plunge the loop deep into the agar during streaking. Flame the loop, make another 2 or 3 streaks at about 60°-90° to the first. Make another two repeats of this procedure: flame and streak. **Check Figure 3**.
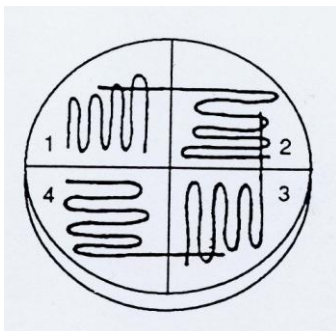

Figure 3

2

Incubation in liquid medium

After inoculation the bacterial culture is stored or incubated in an environment suitable for growth, i.e. development of a large cell population. In liquid medium (broth) the growing population becomes visible as cloudiness (turbidity), but occasionally a surface pellicle or a bottom sediment is formed.

Incubation on solid medium (agar plates)

On solid medium (liquid medium solidified with agar) the bacteria produce a fixed colony of cells that grows to form a visible mass. The separation of single cells from the original inoculum into distinct colonies (colony-forming units, CFU) is a fundamental principle for both isolation, characterization and counting of bacteria in cultures and environmental samples, as will be clear over the next exercises.

Because of a high concentration of water in the Petri plates, condensation forms during incubation. Moisture is likely to cover the agar surface resulting in confluent mass of growth rather than well-separated colonies. To avoid this, Petri plates are routinely incubated bottom-side up.

Report

None

EXERCISE 2: MICROSCOPY OF BACTERIAL CELLS

Introduction

The light microscope
The light microscope (bright-field version) will be used in this and the following exercise to study the bacterial cell (form, size, structural constituents). **Magnification** is the product of those specified for the objective (typically 10X, 40X or 100X) and the ocular (typically 10X). **Resolution** depends on the wavelength of light (λ, e.g. 500 nm for average visible light) and the numerical aperture (A, typically 1.2 for a 100X objective). Maximum resolution (smallest visible object) using the highest magnification (typically 1000X) is: 0.5 λ / A (typically 200 nm or 0.2 μm). This corresponds to the size of a bacterial cell and is often barely enough to visualize the bacteria. A solution is to use the so-called oil-immersion objective (also typically 100X), where a high-grade optical oil (immersion oil) is placed between the specimen (slide with bacteria) and the objective.

Check the textbook for further reading. At the exercise, a thorough introduction to handling the light microscope will be given by the teacher.

The bacterial specimen
There are principally two ways to prepare a specimen (slide with bacteria) for microscopy: If living cells are desired a wet preparation (wet mount) is used, either with or without stain in water or medium. The stain must be non-toxic (vital stain). Alternatively, if killing the cells is acceptable a dry-fixation is often used. Dry fixation can be either an air-drying or heat fixation (about 80-90°C for few seconds)

- *Wet specimen* (vital cell staining). Using wet, unstained mounts and normal transmission light microscopy (low light intensity), bacterial cells can be discerned but often with difficulty. Phase contrast microscopy may here be more useful. The wet mounts are useful, however, to observe e.g. flagellar motility.

- *Dry specimen.* Cellular characteristics are much easier to observe after dry fixation and staining of the specimen. General (universal) stains to detect cell characteristics, e.g. size, form and aggregation, are the alkaline aniline dyes, methylene blue and crystal violet.

Purpose of the exercise
To characterize the bacterial cell using simple methods of specimen preparation (wet/dry mount, staining) in combination with normal, transmission light microscopy. A variety of bacterial species will be compared and emphasis will be made to differentiate cell size and form, flagellar motility, wall characteristics (Gram-reaction) and occurrence of endospore.

Before the exercise 4 different bacterial species have been grown overnight in LB liquid medium to high optical density. At the start of the exercise, the cultures (marked A-D) are thus available as dense cell suspensions in the spent medium.

Materials per team
4 DEMO specimen (4 different species)
4 liquid cultures in vials (4 different species)
4 Petri plates (LB-agar)
Microscopy (slides, cover glass, Methylene blue and Gram stains)

Team size
4 students per team. Each student performs the whole exercise for one bacterial species

Practical work

Week 1

Practicing microscopy

- Install microscope according to teachers instruction. First part of the exercise will offer basic training in handling of specimen, objective lenses, light adjustion, focusing, etc.

- Obtain DEMO specimen with dry, methylene blue-stained bacteria from teacher. Place specimen in microscope. Remember: Bacterial side of slide upwards!

- Use oil immersion objective (100X) directly (without cover slip). Observe form and size of bacterial cells, confirmed by teacher.

- Check other specimen within the team or in the classroom. Remember: Wipe off immersion oil from objective lense after use (and before new specimen is observed).

Preparation of wet mount to observe cell form and motility

- Obtain dense liquid cultures (4 different bacterial species, marked A-D) in small vials from teacher.

- **NB! Before preparing the wet mount, transfer a loopful of the liquid culture to solid LB medium using sterile spread-plating technique (Exercise 1). Mark plates carefully on bottom side by team no. and species code (A-D). Incubate plates at room temperature until Week 2.**

- Using sterile loop, place a loopful of liquid culture on a clean slide for microscopy. Similarly, place a loopful of methylene blue stain **right next** to the bacteria (Do not cross-contaminate the stock solutions of bacteria and stain). Finally, add a drop of water and mix carefully.

- Mount cover glass (if cover glass "floats", remove excess liquid using filter paper) and place specimen in microscope.

- Use dry objectives (10-40X) and look for bacteria in the specimen. Work quickly, since the wet mounts will dry out in the heat from the lamp.

Cell form and motility

- Make observations of 1) Cell form (cocci, rods, possibly bent rods). 2) Cell aggregations (single cells, amorphous clusters, chains). 3) Motility (motile, nonmotile). Check your observations with teacher.

- Save observations in scheme:

	A	B	C	D
Cell form				
Aggregation				
Motility				

Week 2

Preparation of dry mount to observe Gram reaction

- Obtain the incubated LB agar plates containing the four different bacterial species.

- Place a small loopful of bacteria from the youngest part (the edge) of a colony on a clean slide for microscopy. Add a droplet of water to the cell material and make a suspension. Leave it for air-drying (ca. 5 min).

- Pull the slide though a flame (bacterial side away from flame) three times and at one-second intervals. Flame fixation can be tricky… so first, watch the teachers instruction carefully! Leave it for cooling.

Gram stain

- Flood the fixed bacteria with **crystal violet stain** for 1 min. Be careful not to spill the stain on fingers, clothes and on floor! Flush the specimen with water in bottle for 2-3 seconds.

- Flood the bacteria with **iodine solution** for 1 min. Flush the specimen with water in bottle for 2-3 seconds.

- Decolourize the bacteria adding droplets of **ethanol** for 5-15 seconds (while holding specimen at 45° angle) – or until at least part of the specimen has become colorless. Flush the specimen under tap water for 2-3 seconds.

- Flood the bacteria with **safranine** counterstain for 1 min. Flush the specimen under tap water for 2-3 seconds.

- Remove most water (use filter paper to suck water) and leave the specimen for air-drying. Place specimen in microscope – again, bacterial side of slide upwards!

- Use oil immersion objective (100X) directly (without cover slip). Record the observations of violet (Gram-positive) or red (Gram-negative) bacterial cells, confirmed by teacher. Check to see results of other specimen within the team or in the classroom.

- Check and confirm observations from Week 1 of 1) Cell form (cocci, rods, possibly bent rods). 2) Cell aggregation (single cells, amorphous clusters, chains). Save new observations on Gram reaction in scheme:

	A	B	C	D
Cell form				
Aggregation				
Gram reaction				

Report

None, but all results must be collected for evaluation and thorough discussion in Week 3.

EXERCISE 3: CHARACTERIZATION OF BACTERIAL CULTURES

Introduction

Phenotype and genotype
The **phenotype** is the set of structural and functional parameters, often referred to as physiological or biochemical parameters, that characterize the cells within a bacterial culture. In contrast, the **genotype** refer to genetical (DNA- or RNA-related) parameters such as genomic DNA structure, occurrence of plasmid DNA, sequence of ribosomal DNA or RNA, etc., although the latter may specifically be referred to as the ribotype.

Classification and identification
Classification is the term used when the culture is described by these parameters. When the characterization is made using many or important key parameters, classification may be extensive enough to allow for a **tentative identification** of the culture, typically to either genus or species level. Such tentative identification e.g. based on dichotomous keys with a limited number of distinguishing characters such as physiological tests (metabolic activities, growth requirements, etc.), may sometimes be useful. In this exercise we will practise a few such tests and attempt to tentatively identify bacteria using a simple dichotomous key. By comparison, **proper identification** of unknown bacteria, in particular description of completely new genera or species, also requires a characterization of the genotype, see also EXERCISE 4.

Metabolism and growth characteristics
Metabolic activities measured by catalase and oxidase tests are most important for bacterial classification. **Catalase activity** occurs in bacteria thriving in fully aerobic environments, since the enzyme degrades the toxic H_2O_2 metabolite. **Oxidase activity** occurs in bacteria containing cytochrome c (cyt c) involved in the respiratory electron transport.

Many activities can be tested only when the bacteria are transferred to specific rather than universal growth media. In this exercise, we shall test for a couple of activities that require further inoculation using streak plates and liquid media, respectively. **Oxidative/Fermentative activity** refers to "Oxidative" (here defined as O_2 respiring activity, since NO_3^- or other alternatice electron acceptors for respiration are absent in the medium) or "Fermentative" energy metabolism in the bacteria; the two types of energy metabolism can be distinguished in the O/F test (see below), where growth is compared under full and limited O_2 supply, respectively. **Protease activity** occurs in bacteria incubated in e.g. proteinaceous media.

Purpose of the exercise
To characterize cultures of different bacterial species using a number of selected phenotypic parameters. The physiological or biochemical tests include growth activity (colony formation) on universal medium and specific metabolic activity (extracellular enzymes and intracellular enzymes of respiratory metabolism) on selected media. A tentative identification will be made using a simple dichotomous key.

Materials per team
4 LB agar plates (4 different species, prepared in previous EXERCISE 2).
H_2O_2 solution
Glass slides
Tetramethyl-p-phenyldiamine solution
Filter paper
1 Casein agar plate
8 Hugh-Leifson semi-solid agar tubes
Hot vaseline

Team size
4 students per team. Each student performs the whole exercise for one bacterial species

Practical work

Week 2

Purity check of spread plates (universal LB agar medium)

- Obtain LB agar plates incubated in Week 1.

- Check for quality of your streak-plating including purity (single colonies should be present and appear similar in form and size). Check with teacher.

- Observe differences in **colony form** (circular, irregular, etc.), **color** (white, beige, pink, etc.) and **surface characteristics** (slimy, smooth, rough, granular, etc.).

Catalase activity

- From your LB agar plates, transfer a loopfull of bacteria from the edge of a "clean" colony to a glass slide. Mix in a droplet of 3% H_2O_2 (hydrogenperoxide) solution. Observe and record immediate gas formation (O_2 bubbles) as positive reaction.

- Save recorded observations in scheme:

	A	B	C	D
Catalase				
Oxidase				

Oxidase activity

- From your LB agar plates, transfer a loopfull of bacteria with a toothpick from the edge of a "clean" colony to a piece of filter paper. Add a droplet of 1% tetramethyl-p-phenyldiamine solution. Observe and record for purple color formation as positive reaction within 1 min (later reaction is negative).

- Save recorded observations in scheme above.

Protease activity and Oxidative/Fermentative activity

- Transfer bacteria from the edge of a "clean" colony from your LB agar plates to each of the following media:

- *Protease activity*: Inoculate (using sterile needle **with** loop) in a spot on solid casein (milk protein) agar plates. One plate can serve for inoculation of all four species. **Mark by team no. and species code (A-D).**

- *Oxidative/Fermentative activity* (O/F): Inoculate (using sterile needle **without** loop, exchange needle yourself) by deep seeding in two parallel tubes with semi-solid Hugh-Leifson (H-L) agar. Inoculate as deep as the needle allows. Cover the agar surface in one of the tubes with 1-cm deep layer of hot Vaseline, supplied by teacher. **Mark by team no. and species code (A-D).**

- Incubate at room temperature until Week 3.

Week 3

Protease activity and Oxidative/Fermentative activity - continued

- *Protease activity:* Casein agar medium is opaque due to the milk protein. *Protease activity* is observed on the plates as a clear zone around the spot of inoculation where protein hydrolysis has occurred.

- *Oxidative/Fermentative activity*: Semi-solid Hugh-Leifson (H-L) agar medium contains glucose as a C-source for oxidative or fermentative activity. **Aerobic tubes without vaseline**: Oxidative activity can occur thoughout the tube, supported by unlimited O_2 diffusion from the agar surface. **Anaerobic tubes with vaseline:** The vaseline layer added on top of the agar after inoculation reduces the diffusion supply of atmospheric O_2 to the bacteria. While some oxidative activity can occur at the agar surface, only activity by fermentation can occur deeper in the agar medium. Activity is visualized by the yellow color shift (acid reaction from fermentation products) of the pH indicator also contained in the H-L medium. *Oxidative/Fermentative activity* is judged from the absence or presence of yellow color reaction in the agar tubes. Remember: Some bacteria can have both oxidative (respiring) and fermentative energy metabolism, but not at the same time.

- Save recorded observations in scheme:

	A	B	C	D
Protease				
Oxidative				
Fermentative				

Tentative identification

The following simple dichotomous key may be used for tentative identification of the 4 unknown species.

1a. Gram-positive		2
1b. Gram-negative		3
2a. Catalase-positive		4
2b. Catalase-negative		5
4a. Cocci		6
4b. Rods		*Listeria*
4c. Coryneforms (bent rods)		*Corynebacterium/Arthrobacter*
6a. Oxidase-positive		*Micrococcus*
6b. Oxidase-negative		*Staphylococcus*
5a. Cocci		*Streptococcus/Lactococcus*
5b. Rods		*Lactobacillus*
3a. Oxidase-positive		7
3b. Oxidase-negative		8
7a. Oxidative	(non-fermentative)	*Pseudomonas*
7b. Fermentative		*Vibrio/Aeromonas*
8a. Oxidative	(non-fermentative)	*Acinetobacter*
8b. Fermentative		*Escherichia/Serratia/Proteus*

Report

None, but all results from EXERCISES 1, 2 and 3 will be thoroughly discussed in class as led by teacher.

Questions for discussion class

- Discuss why universal medium should be used for purity check of spread plating.

- Give reasons why bacterial colonies can be different in form, color and surface appearance.

- Define catalase activity, oxidase activity and oxidative activity and discuss if these activities are partly coupled or completely independent parameters in bacteria.

- Which products of metabolism and growth activity may cause the yellow color shift in H-L medium (O/F test)? Under O_2 respiring conditions? Under fermenting conditions?

EXERCISE 4: IDENTIFICATION OF BACTERIAL CULTURES

Introduction

To identify an unknown bacterium to the genus and species level, the classification tests performed in EXERCISE 3 may be helpful. Based on so few characters, however, the identification can only be tentative. For a proper identification, many more test characters are most often required.

Commercial identification kits are available for rapid identification of selected groups of bacteria, based on 20-100 biochemical tests characters. API20E is an identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods. The system uses 21 standardized and miniturized biochemical tests and a database.

Each API20E strip consists of 20 microtubes containing dehydrated substrates. Each tube is inoculated with bacterial suspension that reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to a Reading Table. Based on the combination of reactions identification may subsequently be made using the electronic database of the API20E system.

Purpose of the exercise
To identify (unknown) bacteria to the species level using a common, commercial kit (assay system) of biochemical reactions (enzyme activities) and the associated data base.

Materials per team
Agar plates (2 out of 4 different species)
Sterile glass tubes (capped) with 5 ml distilled water
Carlsberg pipettes
Vortex mixer
2 API20E strips
Mineral oil
API20E reagents: TDA, JAMES, VP1 and VP2, NIT1 and NIT2, Zn powder

Team size
4 students per team. Two students perform the whole exercise for one bacterial species.

Practical work

Week 2

Preparation of strip

- Prepare an inoculation box (tray and lid). Distribute 5 ml demineralised water in the tray wells to create humidity during the subsequent incubation.

- Record team no. and species code (see below) on the elongated flap of the tray.

- Remove strip from packaging and place in tray (at a suitable angle, advised by teacher).

Inoculation of strip

- Obtain agar plate from teacher. The species code (numbers 1-4) is marked on the bottom of the plates.

- Transfer a bacterial colony into a glass vial with 5 ml sterile distilled water using sterile technique (loop). Homogenize the cell suspension shortly on a Vortex mixer.

- Fill both tube and cupule (space above tube) of the tests CIT, VP and GEL with the bacterial suspension using a Carlsberg pipette.

- Fill only the tube (and <u>not</u> the cupule) of the other tests.

- Create anaerobiosis in the tests ADH, LDC, ODC, H2S and URE by overlayering with mineral oil supplied by teacher.

- Close incubation box and incubate at 35 ^{o}C.

- **NB! Standard incubation time is 18-24 hours. Teacher will stop reactions at an appropriate time and store the strips at 2°C until readings can be made in Week 3.**

Week 3

Reading of strip

- Obtain strips from teacher. Check Reading Table on how to score negative/positive results. See **Figure 1** next page.

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-ßD-galactopyranoside	0.223	ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase)	colorless	yellow (1)
ADH	L-arginine	1.9	Arginine DiHydrolase	yellow	red / orange (2)
LDC	L-lysine	1.9	Lysine DeCarboxylase	yellow	red / orange (2)
ODC	L-ornithine	1.9	Ornithine DeCarboxylase	yellow	red / orange (2)
CIT	trisodium citrate	0.756	CITrate utilization	pale green / yellow	blue-green / blue (3)
H2S	sodium thiosulfate	0.075	H2S production	colorless / greyish	black deposit / thin line
URE	urea	0.76	UREase	yellow	red / orange (2)
TDA	L-tryptophane	0.38	Tryptophane DeAminase	TDA / immediate	
				yellow	reddish brown
IND	L-tryptophane	0.19	INDole production	JAMES / immediate	
				colorless pale green / yellow	pink
VP	sodium pyruvate	1.9	acetoin production (Voges Proskauer)	VP 1 + VP 2 / 10 min	
				colorless / pale pink	pink / red (5)
GEL	Gelatin (bovine origin)	0.6	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	1.9	fermentation / oxidation (GLUcose) (4)	blue / blue-green	yellow / greyish yellow
MAN	D-mannitol	1.9	fermentation / oxidation (MANnitol) (4)	blue / blue-green	yellow
INO	inositol	1.9	fermentation / oxidation (INOsitol) (4)	blue / blue-green	yellow
SOR	D-sorbitol	1.9	fermentation / oxidation (SORbitol) (4)	blue / blue-green	yellow
RHA	L-rhamnose	1.9	fermentation / oxidation (RHAmnose) (4)	blue / blue-green	yellow
SAC	D-sucrose	1.9	fermentation / oxidation (SACcharose) (4)	blue / blue-green	yellow
MEL	D-melibiose	1.9	fermentation / oxidation (MELibiose) (4)	blue / blue-green	yellow
AMY	amygdalin	0.57	fermentation / oxidation (AMYgdalin) (4)	blue / blue-green	yellow
ARA	L-arabinose	1.9	fermentation / oxidation (ARAbinose) (4)	blue / blue-green	yellow
OX	(see oxidase test package insert)		cytochrome-OXidase	(see oxidase test package insert)	

(1) A very pale yellow should also be considered positive.
(2) An orange color after 36-48 hours incubation must be considered negative.
(3) Reading made in the cupule (aerobic).
(4) Fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.
(5) A slightly pink color after 10 minutes should be considered negative.

Figure 1

13

Reading of strip (continued)

- Start recording the positive or negative reaction results for the **first 20 tests except TDA, IND and VP** as counted from the left of the Recording Scheme handed out by teacher and shown in **Figure 2** below. Continue thereafter with the TDA, IND and VP tests and finish by the 7 tests to the right of the Recording Scheme.

Figure 2

- *TDA test:* Score reaction after addition of 1 drop of TDA reagent.

- *VP test:* Score reaction after addition of 1 drop of both VP1 and VP2 reagents. Read only after 10 min.

- *IND test:* Score reaction after addition of 1 drop of JAMES reagent.

- *NO2 (actually NO_3^- respiration) test*: Score reaction after addition of 1 drop of both NIT1 and NIT2 reagents to the GLU tube. Read only after 5 min. Red reaction color (NO_2^- formation) indicates NO_3^- respiration.

- *N2 (actually denitrification) test:* If negative (colorless) reaction for nitrite production occurs in first step, add 2 mg of Zn powder to the tube. Development of small gas bubbles indicates formation of N_2 by the <u>chemical</u> reduction of NO_3^- by Zn. No bubble formation (absence of NO_3^-) indicates denitrification as performed by the bacteria.

- Finally, teacher will provide data for the remaining tests (OX, MOB, McC and OF-F/OF-O), which must be performed separately.

Identification

Identification can be performed using the Identification Software suuplied by the company.

- Determine the "numerical profile" as follows. On the Recording Scheme (**check Figure 2**), the test are separated into groups of 3 and a value of 1, 2 or 4 is indicated for each. By adding together the values corresponding to **positive** reactions within each group, a 9-digit numerical profile is obtained.

- Compare the 9-digit numerical profile to the list of profiles in Analytical Profile Index. To be demonstrated by teacher.

- Enter the 9-digit numerical profile in the Identification Software. To be demonstrated by teacher.

Report

None, but all results from EXERCISE 4 will be carefully checked and supplemented with questions for discusssion as led by teacher.

Questions for discussion class

- Discuss if the identification performed by the API system is indicative or definite.

- Discuss if the phenotypic characterization performed (e.g. number and type of test parameters) are adequate for proper identification of a new, unknown species.

EXERCISE 5: GROWTH OF BACTERIA

Introduction

How can growth of bacteria be measured?

Measurement of bacterial growth is typically performed to determine the growth of a bacterial population rather than growth of a single bacterial cell. When sufficient nutrients are available, a bacterium will continue to grow and will eventually divide into two new and identical daughter cells. The increased number of cells implies that growth of a bacterial population can be determined from measurements of the cell density at regular intervals.

A. Counting of individual bacterial cells
Various methods have been applied for counting bacteria. Previously, when a microscope was the dominant tool for observing bacteria, a counting chamber (microscope slide with an engraved grid) was used for counting individual cells. The technique requires experience and there are several potential risks for obtaining erroneous bacterial numbers. For example, small bacterial cells may be overlooked, dust particles may be mistaken for bacteria, and living and dead cells cannot be distinguished.

Today, electronic particle counters have replaced microscopic counting of bacteria. A typical cell counting is done by flow-cytometry, a technique by which the bacterial culture is diluted in a thin glass tube detected by fluorescence[1] techniques. A flow-cytometer can count from 100 to 30,000 cells per second. If different fluorochromes targeting different bacterial groups are used, the number of cells in each bacterial group can be enumerated simultaneously.

B. Counting of colony-forming units
The most widely technique for counting bacteria, however, is still plate counting. In the plate counting method, an exact volume of the bacterial culture is inoculated (layered) on top of an agar medium which contains an adequate growth medium. Each bacterium will begin dividing and hereby form colonies. Thus, each colony represents one bacterium. The technique is simple, inexpensive and usually acceptable accurate for most purposes. As colonies rather than single bacterial cells are counted, bacterial numbers obtained by plate counts are named colony-forming units, or just CFU.

In order to obtain a reproducible number of bacteria by plate counts, the bacterial density on a tradiional 9 cm diameter agar plate should be between 30 and 300 colonies. With a lower number, the statistically uncertainty becomes too high. When the number of colonies exceeds about 300, the individual colonies are difficult to enumerate, and colonies close to each other can merge and maybe counted as one colony. Moreover, with a high density, nutrients may become limiting. To obtain a satisfactory number of colonies on the agar plates, the bacterial culture has to be diluted. Typically a serial dilution series is made, e.g., 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} etc, and a volume from each dilution is inoculated onto agar plates.

C. Measurements of bacterial biomass: Turbidity
If the generation time of a bacterium (time required for a complete cell division) rather than the actual number of produced bacteria is the aim of a study, a fast and simple analysis of the turbidity of the culture can be applied. A subsample of the bacterial culture is transferred to a spectrophotometer, in which the light absorption at 600 nm is measured. The more cells in the culture, the higher the absorption of light will be. The turbidity of a bacterial culture does not only depend on the density of the cells, but also on their size and shape. This means that a turbidity measurement often correlates better with the cell biomass than the exact cell number.

---

[1] Fluorescence: Some substances absorb light of a specific spectral region, e.g., blue, and instantaneously emit light in another spectral region, e.g., yellow. Bacteria are typically stained with fluorescent dyes that bind to double-stranded (ds) DNA. When the stained bacteria are exposed to blue light, all cells with ds-DNA will emit green or yellow light and are easily detectable.

A turbidity analysis requires a certain amounts of bacteria. If the cell density is below about 10^6 cells per ml, the method cannot be used. On the other hand, if the cell suspension is too high, the sample must be diluted before analysis. The turbidity measurement is also called an optical density measurement, or OD for short. Turbidity readings on a spectrophotometer will range from 0.001 to about 1.000. Values above 1.000 should not be used due to lack of linearity.

D. Measurements of bacterial biomass: Content of DNA
The DNA content in bacteria makes up from 10% (large bacteria) to 20% (small bacteria) of the dry weight of the cells, but each bacterial species will have a relatively constant DNA content. This means that the DNA content per volume in a bacterial population can be used as a measurement of the bacterial biomass, and that changes in the DNA content can be used to measure the growth of the bacterial population. DNA can easily be quantified with a number of different fluorescent stains. A popular fluorochrome for detection and quantification of DNA is SYBR Green1, which produces highly fluorescent DNA derivatives that easily can be quantified in a fluorescence spectrophotometer.

Calculation of bacterial growth rates

Bacterial growth is not completely adjusted to logistic growth, as they usually have an initial lag phase during which they produce new enzymes to optimize their growth. After this lag phase, each bacterium will divide into two daughter cells. This leads to exponential growth:

$$N = N_0 2^n$$

where N = final number of bacteria (equals the carrying capacity)
N_0 = initial number of bacteria and
n = the number of bacterial generations

Check section 6.6 in Brock Biology of Microorganisms (12th edition)

Exponential growth can be illustrated as a straight line in an X-Y plot with the culture age on a linear x-axis and the number of bacteria (or Optical Density, OD) on an exponential y-axis.

The equation $N = N_0 2^n$ can be transformed in several ways. Often the most interesting information will be the generation time during exponential growth. The generation time is determined from slope, α, of the line in a semi-logarithmic plot using the equation $\alpha = \log 2/g$, or $g = 0{,}301/\alpha$.

Purpose of the exercise

The purpose of this exercise is

- to apply three independent techniques for measuring bacterial growth (optical density (OD), number of CFU and content of DNA)
- to demonstrate exponential growth of two bacterial species (*Serratia marcescens* and *Pseudomonas fluorescens*)
- to calculate generation times of the two bacteria when grown at laboratory conditions

Experimental background and setup

S. marcescens and *P. fluorescens* are commonly found bacterial species in nature, where they degrade organic matter (are chemoorganotrophs). In the laboratory, the bacteria grow well in liquid LB-broth (Luria-Bertani broth), which contains a mixture of proteins, amino acids, carbohydrates and mineral salts. When grown on agar plates with LB in solid form, *P. fluorescens* forms grayish colonies, while *S. marcescens* produces red colonies due to the compound prodigiosin.

Before start of the exercise, the two bacteria have been inoculated into liquid LB media at one hour intervals for 5 hours, followed by incubation on a shaking table at 28°C. This means that there at start of the exercise will be bacterial cultures that have grown for 1, 2, 3, 4 and 5 hours.

Materials per team

- Two flasks with either *S. marcescens* (culture 1) or *P. fluorescens* (culture 2)
- Automatic pipettes and sterile tips
- 1.5 ml eppendorf tubes for dilution, plating and staining with SYBR Green1
- Microcuvettes for OD measurements
- SYBR Green1 solution
- Four-way transparent 4 ml cuvettes for fluorescence measurements
- 0.9% NaCl for dilution of cultures
- PBS buffer for fluorescence assay
- 4 Petri dishes with LB agar
- Drigalsky spatula and ethanol for sterilization
- Ice bath

Team size: 4 students per team. Teams and cultures

Team No.	Culture No.	Culture age
1	1	1 h
2	1	2 h
3	1	3 h
4	1	4 h
5	1	5 h
6	2	1 h
7	2	2 h
8	2	3 h
9	2	4 h
10	2	5 h

Practical work

Week 4

Start

When the bacterial cultures have grown for exactly 1, 2, 3, 4 or 5 hours, the cultures will be brought to the laboratory in an ice bath. Find your cultures and place them immediately in a small ice bath at your working bench. Prepare measurement of OD, fluorescence assay and plate counts.

1. OD measurements

Transfer 1 ml sample of the bacterial culture into a microcuvette. Measure OD at 600 nm. Remember to check that the spectrophotometer is zero-adjusted with a LB solution. Write the result in the table on page 21.

2. Plate counts

Start diluting the cultures, as the cell density otherwise will be too high. The dilution is shown in the figure below and involves the following steps:

- Label 7 eppendorf tubes with numbers 1 to 7.
- With a sterile pipette tip, transfer 900 µl 0.9 % NaCl into all tubes (Tube 1 to 9)
- Transfer 100 µl of the original culture into Tube 1 (dilution 10^{-1}). Mix the sample on a whirly mixer.
- Transfer 100 µl of the sample in Tube 1 into Tube 2 (dilution 10^{-2}). Mix the sample.
- Repeat the dilutions until dilutions of 10^{-7} (Tube 9) is reached.
- With a sterile pipette tip, transfer 100 µl of the sample in Tube 5 and Tube 7 into each of two Petri dishes. Spread the sample evenly on top of the agar with a Drigalsky spatula.
- Incubate the plates at room temperature until next week.

| Dilution: | 10^{-1} | 10^{-2} | 10^{-3} | 10^{-4} | 10^{-5} | 10^{-6} | 10^{-7} |
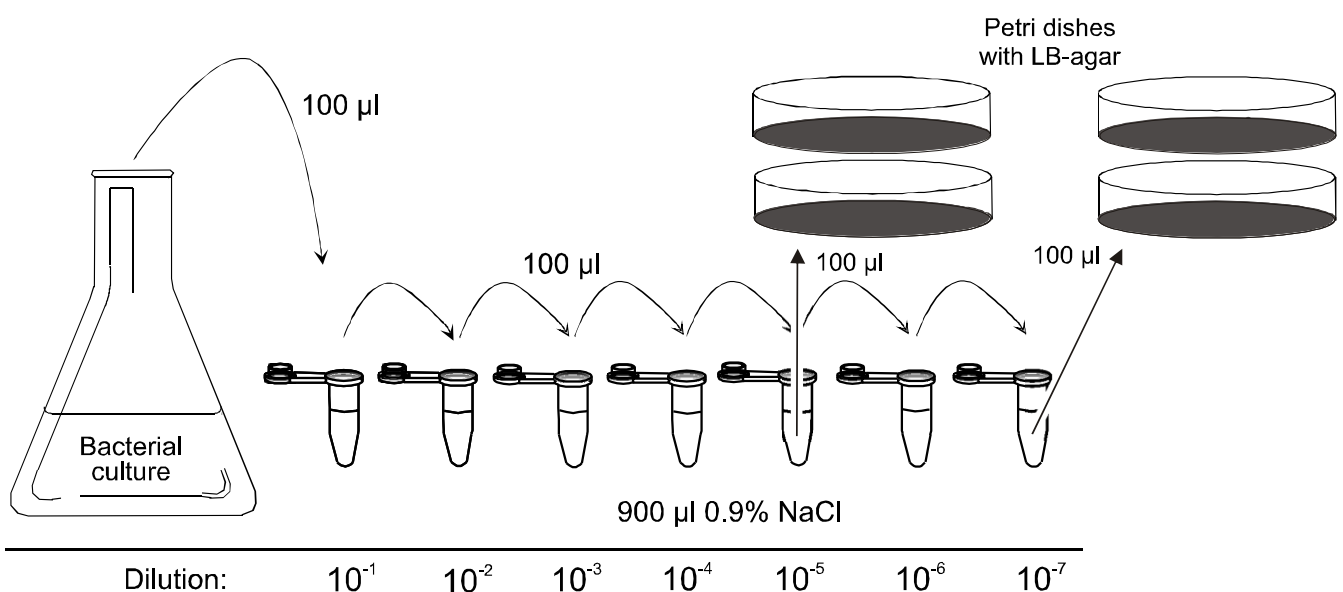
3. Fluorescence assay of bacterial DNA

The bacterial DNA content is measured with the fluorochrome SYBR Green1. The fluorochrome combines with DNA and emits yellow light (520 nm) when illuminated (excited) with green light (497 nm). The fluorescence signal is measured in a fluorescence spectrophotometer.

- Before measuring DNA in the bacteria, the growth media (LB broth) has to be removed from the bacterial cultures by washing and centrifugation. LB broth contains dissolved DNA that will interfere with the analysis of DNA in the bacteria. The fluorescence analysis is done as duplicate measurements relative to a blank control without bacteria. The blank sample is treated like to bacterial samples to make a similar treatment of all samples.

- Label 3 eppendorf tubes 1, 2 and 3 and add 1.3 ml PBS to each tube.
- Transfer 100 µl *undiluted* culture to tube 2 and 3 and mix. Tube 1 is a blank control and shall not be added bacteria.
- Bring tube 2 and 3 to the centrifuge and place them in the rotor. Remember to place the tubes in an order that will keep the centrifuge rotor balanced. You can include tubes from other teams.
- Spin the tubes at 6000 *g* for 5 min. This will separate bacteria from the LB broth.
- Suck up all of the supernatant from tube 2 and 3 with a pipette. Avoid removing the pellet.
- Add 1.3 ml PBS buffer to tube 2 and 3, mix lightly
- Repeat the centrifugation. After centrifugation, remove the supernatant.
- Add 1.3 ml PBS buffer to tube 2 and 3 and mix the tubes thoroughly.
- Finally, add 15 µl SYBR Green1 solution to all 3 tubes and mix again.
- After 5 min (or longer), transfer the samples to each of 3 four-way transparent 4 ml cuvettes with a pipette and measure the fluorescence intensity.

Settings for the fluorescence detector should be: excitation 497 nm, emission 520 nm and sensitivity factor = 10.

Calculate the DNA concentration in the samples from DNA standard curve below. Use the regression Y (DNA) = 1.163 + 1.908 X (fluorescence). The standard curve is made from herring sperm.

Add the calculated mean DNA content (average of the two samples – the content in the blank control) in the table on next page.

Regression: Y = 1.163 + 1.908 X (r^2 = 0.9994)
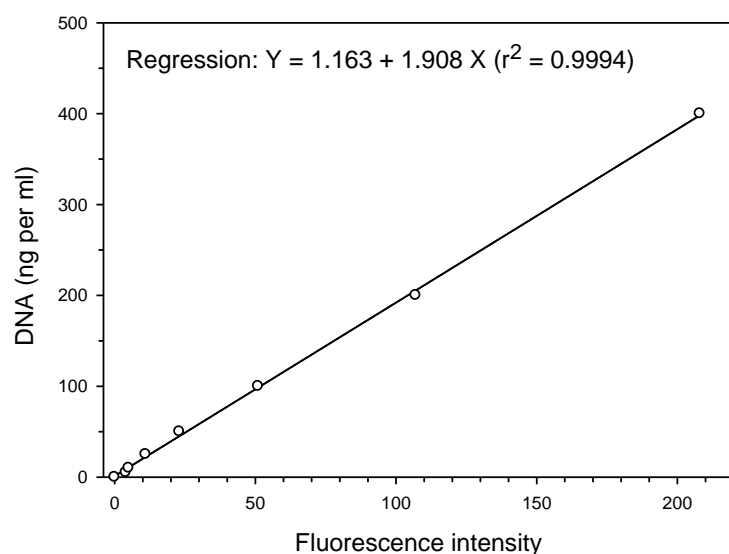
Week 5

Number of colonies on the Petri dishes
Count the number of colonies (CFU) on the Petri dishes from each period. Write the numbers in the table next page. Convert colonies per plate to bacteria per ml.

Example:
23 colonies at a 10^{-7} dilution on a Petri dish equal 23×10^{8} colonies or bacteria pr. ml, as the inoculation volume was 0.1 ml (100 µl).

Table: OD, DNA content and number of colonies of *S. marcescens and P. fluorescens*

Incubation time (hours)	OD	DNA (ng/ml)	Colonies per Petri dish		Number of colonies pr ml	
1						
2						
3						
4						
5						

Report

1. Exponential growth and generation times

Contact the other teams and collect results for CFU, OD and DNA content at 1, 2, 3, 4 and 5 h. Make graphs and calculations as indicated below.

A: Did the bacteria grow exponentially?

(1) Make graphs showing CFU, OD and DNA content vs. time at linear x and y scales as shown in the upper graph in the figure at right.

(2) Make graphs showing CFU, OD and DNA content vs. time at linear x scales and logarithmic y scales as shown in the lower graph in the figure at right.

- Do the graphs indicate exponential growth?

B: What were generation times of the bacteria?

Use results from the semi-logarithmic plots to determine the generation time *g* of the bacterial cultures. The generation time can easily be calculated from the equation *g = 0.301/α* (*α* is the slope of the lines).

- Calculate the slope *α* of the semi-logarithmic graphs from:
  $\alpha = (\log Y_2 - \log Y_1)/(X_2 - X_1)$.

  Y_1 and Y_2 are values of OD, CFU or DNA at X_1 and X_2 hours, e.g. 1 and 5 hours.

- Calculate the generation time *g* from the equation *g = 0.301/α*

- Did generation times based on CFU, OD and DNA measurements compare with each other?

- Which procedure (CFU, OD or DNA content) produces the most reliable results?

2. Questions

- Which method would you choose to prove that bacteria grow exponentially?

- Calculate the DNA content per bacterium in units of 10^{-xx} grams per cell. Did the DNA content change during the 5 h growth period? Is the DNA content expected to vary during growth?

- Do the results indicate that the two bacteria have different "carrying capacities" (reach different final densities in the cultures)?
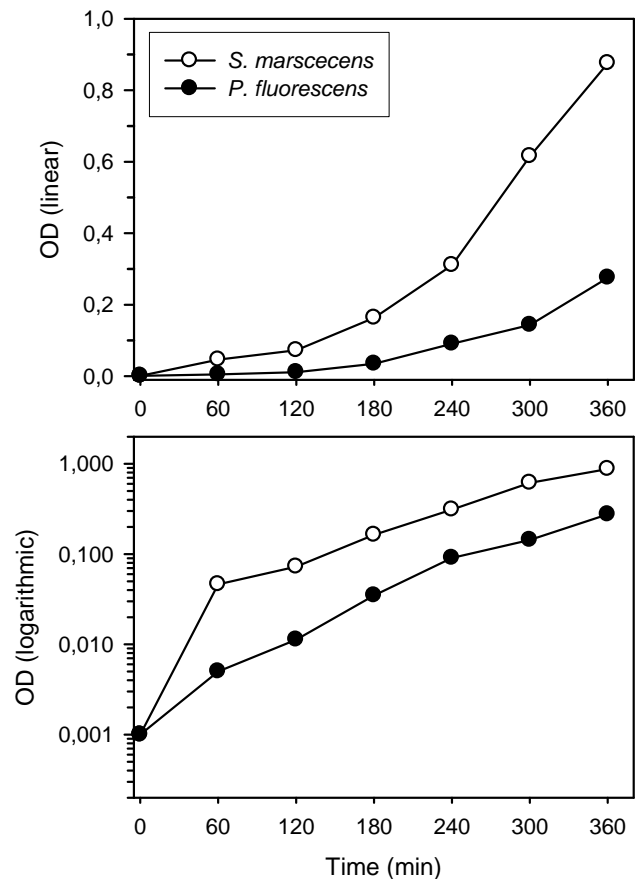
Growth curves of *S. marscecens* and *P. fluorescens* during a 6 h period. Linear and semi-logarithmic plots are shown.

EXERCISE 6: INFLUENCE OF ENVIRONMENTAL EFFECTS ON GROWTH OF BACTERIA

Introduction

In addition to supply of organic and inorganic nutrients, growth of microorganisms is influenced by many external factors. In this exercise we will examine how temperature, pH and salinity influence the growth of different bacteria.

Temperature

Temperature probably is the single most important environmental factor to microbial life, as it controls both growth rate and survival of microorganisms. Through the evolution, microbes have adapted to a wide range of temperatures, and only very extreme environments are today devoid of bacteria and fungi. In the textbook, Chapter 6, you can read more about relations between temperature and microbial growth, and definitions of the bacterial temperature categories *psychrophilic*, *mesophilic*, *thermophilic* and *hyperthermophilic*.

The optimum temperature for bacterial growth can be determined by measuring the growth rate of cells in the exponential phase at different temperatures, e.g., 10, 20, 30, 40 and 50°C. At short time intervals, samples of the cultures at each temperature are taken and the number of cells (using OD or CFU) is determined. If a large range of temperatures are to be tested for different bacteria, this will end up with a very large number of samples.

In attempt to simplify the temperature test and still get trustworthy results, incubation of bacterial cultures at different temperatures for 2 to 7 days, followed by a turbidity measurement, has been successfully used. Potential sources of error in this setup may be:

- *Differences in growth rates.* Even though two bacterial species belong to the same temperature category, e.g., both being mesophilic species, they do not necessarily have identical growth rates. This may mean that the final turbidity (OD) will differ between the two cultures.
- *Differences in growth efficiency.* Bacterial species typically differ with respect to growth efficiency, that is, the capacity to produce new cells from a certain amount of nutrients. A bacterial species with a high grow efficiency will typically produce more cells than a species with a low growth rate, leading to different turbidities within a time period.
- *Oxygen limitation.* In a fast-growing culture, the oxygen consumption may introduce anoxic conditions, if oxygen is used faster than it can diffuse into the media. This may limit the bacterial growth relative to slow-growing species that maintain a satisfactory oxygen level in the medium.

pH

Most bacterial species have optimum growth at a neutral pH level, as this corresponds to the intracellular pH level of 6 to 8. Maintenance of a relatively constant intracellular pH level is necessary for the function of many enzymes, for a proper function of the energy-producing electron transport and for the stability of various macromolecules. A stable pH level inside the cells is typically sustained by pumping H^+ and OH^- ions across the cell membrane.

A few bacterial species have adapted to very acidic or alkaline environments. In acidic soils and mine galleries, pH can be as low as 1 and here *acidophilic* bacteria thrive. Acidophilic bacte-

ria have developed special acid-tolerant cell membranes and they often take advantage of the low pH in their ATP production by modified electron transport systems. Some microorganisms, such as *Lactobacillus*, utilize a low pH as an inhibitory agent against other bacteria and produce acidic substances like lactic acid and other carboxylic acids.

In alkaline soils and lakes, where pH may reach 12, *alkaliphilic* bacteria occur. Like the acidophilic bacteria, alkaliphilic bacteria have developed special cell membranes and enzymes than can resist the extreme pH conditions. Since the concentration of H^+ ions is very low at high pH, it can be difficult for alkaliphilic bacteria to maintain a proton motive force based on H^+. Therefore some alkaliphilic bacteria utilize Na^+ instead of H^+ in the electron transport for production of ATP.

The ability of microbes to adapt to extreme pH values has been beneficial to human life. Examples are preservation of foods by treatment with lactic bacteria (lowering of pH) and industrial production of proteases and lipases from alkaliphilic bacteria. The enzymes are important additives in household detergents.

Salinity

A living cell can only function optimally if it has a proper composition of inorganic ions. Most bacteria live in environments with an ion composition very different from that in the intracellular fluids. Since many ions can move freely across the cell membrane, this transport will eventually destroy the correct osmotic properties of the cell. In order to maintain a correct ion composition in the cytoplasm, bacteria have transport enzymes that take up or excrete ions to establish a correct ion composition.

Bacteria differ considerably with respect to tolerance to high salinities, probably because they possess a variable capacity to excrete intruding ions. The capability among bacteria to tolerate salts vary from non-halophilic species (grows at salinities up to about 1-2%) to extreme halophilic species than survive >30% NaCl. Most bacteria in natural environments grow well at salinities up to about 3% NaCl.

The inhibitory effect of salts on microbial growth has been exploited by humans to preserve food since ancient times, but even today, salts are important preservatives in the food industry.

Purpose of the exercise

The purpose of this exercise is
- to demonstrate how the environmental factors impact the growth of different bacteria
- to test how temperature, pH and salinity may control growth of specific bacteria
- to learn how different preservation techniques can reduce growth of bacteria in food

Experimental background and setup

- Temperature assay: The bacterial species *Pseudomonas sp.*, *Bacillus stearothermophilus* and *Escherichia coli* will be examined*.* Each of the three bacteria belongs to one of the following temperature categories: psychrophilic, mesophilic or thermophilic.
- pH assay: The bacterial species *P. fluorescens, Serratia marcescens* and *Alicyclobacillus acidocaldarius* will be examined. *P. fluorescens and S. marcescens are* expected to grow best at neutral pH, while *A. acidocaldarius* is acid-tolerant.
- Salinity assay: The bacterial species *P. fluorescens, S. marcescens* and *A. acidocaldarius* will be examined in media with increasing salinity (NaCl concentration).

Materials per team

- Temperature assay: 21 tubes with 10 ml L-broth
- pH assay: 15 tubes with L-broth at pH 3, 5, 7, 9, 11
- Salinity assay: 15 tubes with L-broth at 0, 1, 3, 5, 10% salinity
- Bacterial cultures (see also table below):
  Pseudomonas sp., Bacillus stearothermophilus, Escherichia coli, P. fluorescens, S. marcescens (all grown in LB broth), and *Alicyclobacillus acidocaldarius* (grown in LB broth at pH 4)
- Pipettes and sterile tips and racks
- PF-11 portable photometer (week 5)

Team size: 4 students per team

Teams and cultures

Temperature assay (teams 1, 2. 3 and 4):

	2°C	10°C	20°C	30°C	40°C	50°C	60°C
Pseudomonas sp.							
B. stearotermophilus							
E. coli							

pH assay (teams 5, 6 and 7):

	pH 3	pH 5	pH 7	pH 9	pH 11
P. fluorescens					
S. marcescens					
A. acidocaldarius					

Salinity assay (teams 8, 9 and 10):

	0%	3%	5%	10%	15%
P. fluorescens					
S. mascescens					
A. acidocaldarius					

Practical work

Week 4

- Find the bacterial cultures to be used and tubes with the different media.
- Arrange the tubes correctly in racks and mark the individual tubes with team number and treatment.
- With a pipette and 100 µl sterile tips, add bacteria to the different tubes. You can use the same tip for inoculation of all tubes with the same bacterium. Cap the tubes immediately after addition of culture.

Incubation

Temperature assay: The teacher will place the tubes in incubators at temperatures at 2 to 60°C.
pH and salinity assays: Tubes with *P. fluorescens* and *S. marscecens* are incubated at room temperature in the laboratory, while tubes with *A. acidocaldarius* are incubated at 60°C.

Week 5

- Mix the tubes on the whirly mixer to suspend the bacteria

- If you have written numbers etc. on the lower 1/3 of the tubes, wipe off the writings

- Remove condensed water on the tubes with paper tissue (only on tubes from temperature assays at 2 and 10°C)

- Measure OD of the cultures using the PF-11 portable photometers with filter number 5 (605 nm wavelength):

  1. Select NANOCOLOR with the M bottom. Confirm with left arrow-marked bottom.
  2. Place a blank tube (LB broth without bacteria) in the measuring cell and press Null/Zero 2 times.
     In the pH assay, adjust the photometer to zero with LB broth for each pH value (pH 3, 5, 7, 9, 11), as the colour of the medium changes with the pH.
  3. Place the incubated tubes in the measuring cell, one at a time. Press M to measure the Absorption and note the different absorption values.
  4. Arrange the results in a table like in the tables on the previous page and exchange results with the other teams.

Report

1. Make a bar graph of the measured growth (OD values; y-axis) vs. treatment (increase in temperature, pH or salinity; x-axis).
Get results from the other teams and make bar graphs for temperature, pH and salinity.

2. What were optimum conditions with respect to temperature, pH and salinity for the different bacteria?

3. Explain why *Alicyclobacillus acidocaldarius* can be a problem in production of yoghurt and other milk products.

4. Which temperature, pH or salinity groups can the bacteria be referred to (check chapter 6 in the textbook)?

5. Consider potential sources of error that may have influenced the obtained results.

   Hints: Was there an oxygen limitation? Do all bacterial species grow equally fast and do they convert the same amount of food into bacterial cells?

EXERCISE 7: INHIBITION OF BACTERIAL GROWTH BY UV RADIATION

Introduction

The most widespread and inexpensive procedure for controlling microbial growth is application of heat. Above 100°C most macromolecules denature, a process by which the molecules loose their structure and ability to function. The antimicrobial effect of heating is used in *autoclaving* where the material is heated to about 115°C for 15 min to kill bacteria and fungi.

An alternative technique to heating is use of electromagnetic radiation such as microwaves, X-rays and UV light. The antimicrobial effect of radiation can be caused by heating of the material, e.g., by exposure to microwaves, or destruction of vital molecules, e.g., damage of DNA by UV radiation. UV radiation is commonly used for disinfecting larger surfaces such as laboratory and flow benches, but it is also be used for sterilization and purification of water in laboratory water supplies such as Millipore water systems.

Research on UV effects in microorganisms has been received an increasing attention within the last 15 years due to depletion of the ozone layer and its environmental effect. As an example, it is now known that many bacteria in Antarctic waters suffer DNA damage due to the intense solar radiation. However, most of the Antarctic bacteria (and many bacteria in general) produce enzymes that can repair the DNA damage during the night. Thus, the expected increased inhibitory effect of solar radiation on the microbial populations is smaller than assumed. In laboratory experiments it has further been shown that some bacteria begin producing UV-absorbing pigments when exposed to UV light as a precaution against radiation damage.

Decimal reduction time

When testing a controlling effect on bacteria in the laboratory, the number of cells surviving the treatment will typically be determined by plate counts (colony-forming units, CFU). As a precaution, one should keep in mind that some bacterial cells may still be viable although they do not produce CFU on the agar media. This is especially important when dealing with pathogenic bacteria.

If effects of different treatments are to be compared, the controlling effect is often expressed as the *decimal reduction time* or *D*. *D* is the time required for a 10-fold reduction in population density at a given treatment. The *decimal* reduction means that when *D* is tested at different periods of heating or UV radiation, relationship between *D* and heat or UV will be an exponentially decreasing relation.

The effect of exposing a culture of *S. marcescens* with UV radiation over increasing periods of time is shown in the figure at right. The initial number of cells of 98×10^6 was reduced to 8.2×10^3 after 70 sec of radiation. *D* was determined from the regression line during the initial 40 sec period and was found to be 23 sec. See next page for details of the calculation.
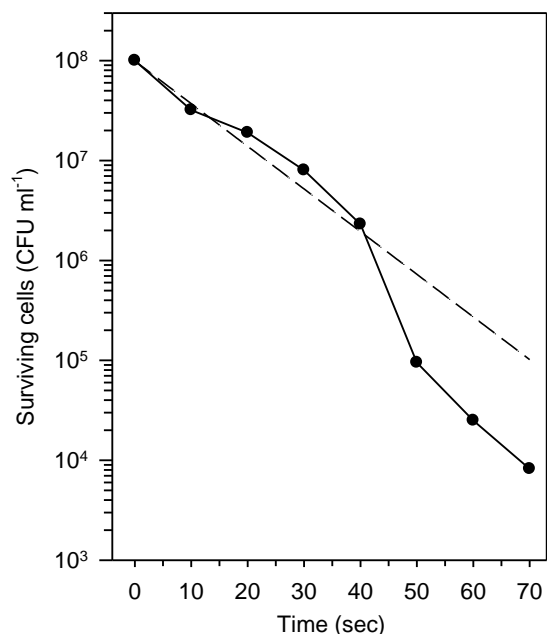
Effect of UV radiation on survival of *Serratia marcescens.* Dashed line indicates fitting to a linear regression.

Here are the calculations: Using Excel, the equation for the regression (the dashed line in the figure on the previous page) was calculated to be

$$y = (98 \times 10^6) \, e^{-0.098\,x} \quad (y = \text{cell density and } x = \text{UV radiation time})$$

Decimal time for a reduction from all cells (= 98 x 10^6) to 10% cells (= 98 x 10^5) is
$98 \times 10^5 = (98 \times 10^6) \, e^{-0.098\,x}$ or $0.1 = e^{-0.098\,x}$.

This means that ln 0.1 = -0.098 x which leads to **x = 23 sec.**

Thus the decimal reduction, *D*, time was 23 seconds in the experiment.

Note that some cells survived even 70 sec of UV radiation. A likely reason for this might be that these bacteria were covered by other cells and received a lower total dose of radiation.

Purpose of the exercise
The purpose of the exercise is
- to determine the effect of UV radiation on survival of the bacterium *Serratia marcescens*
- to calculate the decimal reduction time, *D*, for *S. marcescens* when exposed to UV radiation

Experimental background and setup
In the experiment, a diluted culture of *S. marcescens* is spread on a number of Petri dishes that are exposed to UV light for periods of 5 to 35 seconds. After the radiation, the plates are incubated until next week. The number of CFUs on the plates represents the surviving cells. When the CFU number is plotted against the exposure time, the decimal time can be determined.

Materials per team

- Culture of *S. marcescens* in eppendorf tube
- 0.9% NaCl for dilution of the culture
- Automatic pipettes, sterile tips and empty eppendorf tubes
- 8 Petri dishes with LB agar
- Drigalsky spatula and 96% ethanol
- Protection goggles and gloves
- UV lamp. Must be turned on 30 min before use. For radiation of the Petri dishes, distance between the lamp and the dishes should be about 90 cm.
- Stopwatch

Team size: 4 students per team

Practical work

Week 4

1. Dilution of *S. marcescens* culture

Before *S. marcescens* can be spread on the pates, the culture must be diluted to 10^{-6}.

- Label 6 eppendorf tubes with number 1 to 6.
- With a sterile pipette tip, transfer 900 µl 0.9 % NaCl into each eppendorf tube.
- Mix the original *S. marcescens* culture for a few seconds on a Whirley mixer to ensure that the cells are evenly distributed in the tube.
- Transfer 100 µl of the original culture into Tube 1 (= dilution 10^{-1}). Mix the sample.
- Transfer 100 µl from Tube 1 into Tube 2 (= dilution 10^{-2}). Mix the sample.
- Continue the dilution series until dilution 10^{-6} is reached in Tube 6.

2. Spreading of the 10^{-6} diluted *S. marcescens* culture on LB agar plates

- Label 8 Petri dishes (with LB agar) with number 1 to 8. Also, remember to add your team number.
- With a sterile pipette tip, transfer 100 µl of the sample in Tube 6 onto each of the 8 Petri dishes. Spread *immediately* the sample evenly on top of the agar with a sterile Drigalsky spatula. Sterilize the spatula between spreading the culture on the plates.

3. UV radiation of Petri dish number 1 to 7

The Petri dishes shall be exposed for 5 to 35 seconds under the UV lamp, one dish at a time. Dish number 8 is a control and must not be exposed.

- Use the stopwatch to keep track of the time. Use goggles and plastic gloves if you wish.

- Work fast and accurate when you expose the plates to UV light: Place one dish at a time under the lamp and immediately remove the cover. Watch the stopwatch. After the exposure, remove the dish quickly and immediately place the cover on top.

The following exposure times are used:

Petri dish number	Exposure time (sec)
1	5
2	10
3	15
4	20
5	25
6	30
7	35
8	Control. No exposure.

- Finally, the plates are incubated until next week. The teachers will check growth of CFU on the plates after 3 days and then place the plates in the refrigerator.

Week 5

- Count the number of CFU on each of the 8 Petri dishes. Assume that one CFU represents one bacterium.

Report

1. Transfer the results from dish 1 to 8 to an Excel spreadsheet on your computer. Plot number of bacteria (Y axis) against irradiation time (X axis) in an "XY punkt" diagram. Change scale on Y axis to logarithmic units.

2. Let Excel determine an exponential regression line (*Danish*: tendenslinie). Give equation of the regression in the report. Does the regression line match the course of the decline in cell numbers?

3. Determine the decimal reduction time *D* in your experiment.

4. If you do not have Excel at hand in the laboratory, you can make a manual plot in a semi-logarithmic diagram on paper, or use your calculator for the calculations.

5. In the report, discuss the following:
    - The mechanisms causing cell death by UV radiation
    - How cells can reduce damages from UV radiation

EXERCISE 8: BACTERIOPHAGES

Introduction

Bacteriophages constitute a group of viruses that attacks bacterial cells. The bacteriophage (or just the phage) injects its nucleic acid into a host bacterium. If the phage is virulent it takes over the metabolism of the bacterial host and re-directs it to produce new phages. Often the host is killed by the phage infection; hence phage attack can constitute an important mortality factor for bacteria in Nature or in industrial production systems.
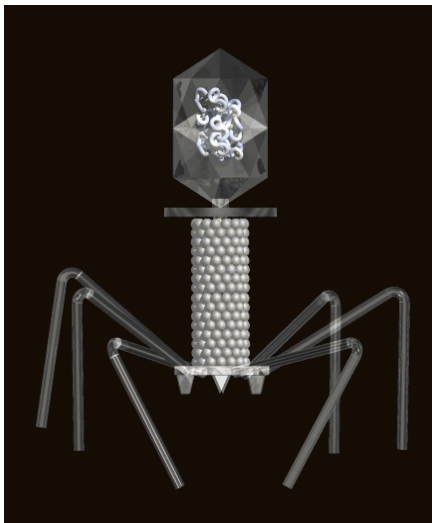
The bacteriophage T4 used in this exercise; a very complex phage that attacks *Escherichia coli*

In natural environments, such as sea water, as much 10 mio virus particles can be found in 1 ml and as much as 70% of the bacteria can be infected by phages. Therefore an understanding of ecosystem function including turn over of nutrients requires a detailed analysis of phage – host interactions. In the biotechnological industry, specific bacterial cultures are frequently used as starter cultures for fermentation processes, e.g. for cheese and wine production. Bacteria are even used in industry to produce specific enzymes or metabolites. Obviously, phage attack in such production systems can lead to severe economic losses and much attention is focused on preventing or combating these attacks. In other cases phage attacks are useful seen from a human perspective. For example, researchers are considering using phages as an alternative to antibiotics to combat pathogens in systems as different as human wounds and aquaculture production ponds. This approach is known as phage therapy.

Finally, phages can be important vectors for horizontal gene transfer between bacteria. These transfer mechanisms are known as general and specialized transduction. Studies of horizontal gene transfer are in particular accentuated by the increasing spread of antibiotic resistance genes and by the risk assessments that need to be performed prior to the release of genetically modified microorganisms to solve specific problems in the environment, for example biological control of harmful microorganisms or biodegradation of pollutants.

The total number of viruses in a sample can be determined by staining the nucleic acids with a fluorescent stain and visualizing the stained virus particles in a fluorescence microscope. This approach is dealt with in the lectures. The number of phage particles attacking a specific bacterium is determined by the plaque-assay, which is described below. Please note, that by a plaque assay you can only determine the

number of phages attacking the host bacterium you add to the assay, in our case an *E. coli* strain. In principle the sample could contain other phages attacking e.g. lactic acid bacteria. In order to detect phages with other host bacteria we would have to make additional plaque assays with alternative host bacteria.

Aim of the exercise

The aims of the exercises are:
- to demonstrate and train use of plaque assays
- to use plaque assays to follow a T4 phage infection of *Escherichia coli* by a one step growth experiment.
- to determine burst size and latent period (see below) that are characteristics for a specific phage infection

Experimental background and set up

In this exercise you prepare a dilution series of a T4 phage stock solution and determine the titer (phage concentration) by the plaque assay. The principle of the plaque assay is explained in the textbook, section 10.4 and shown in figure 10.6. In brief, the bacteriophages in the sample infect the host bacterium, which grows in a thin top-layer of an agar plate. In the absence of phages, the bacteria will form a confluent layer on the agar plate. However, if a cell is infected with a virulent phage it will lyse. This releases new bacteriophages that will infect the neighbouring bacterial cells. Is this way an infection center is formed that can be seen as a clear spot on the agar plate. These spots are known as "plaques" and you assume that a single phage forms a single plaque. The number of plaques on a given plate is used to calculate the number of plaques-forming units (PFU) per ml.

In this exercise you also follow a single round of phage amplification, se the textbook figure 10.9. Initially you inoculate a culture of the host bacterium, *E. coli* strain BAU. After 45 min the culture will be in the exponential growth phase. Then you start the one step growth experiment by adding phages. You have fewer phages than bacteria per ml so that the probability that one bacterial cell is infected by more than one phage is minimized. Due to the high number of bacteria, the added phages rapidly adsorb to their host bacteria. This means that the infection cycles start at approximately the same time.

Five minutes later the infected culture is diluted 10,000-fold to reduce adsorption of new phages after that time point. You then follow the course of the infection by taking out samples and running a plaque assay with 5-min intervals during the next 40 min. You can then determine how long time it takes before new phages are produced from infected cells (typically 25 min for T4) and how many new phages that are produced per infected bacterial cell (burst size; typically 50-100 for T4).
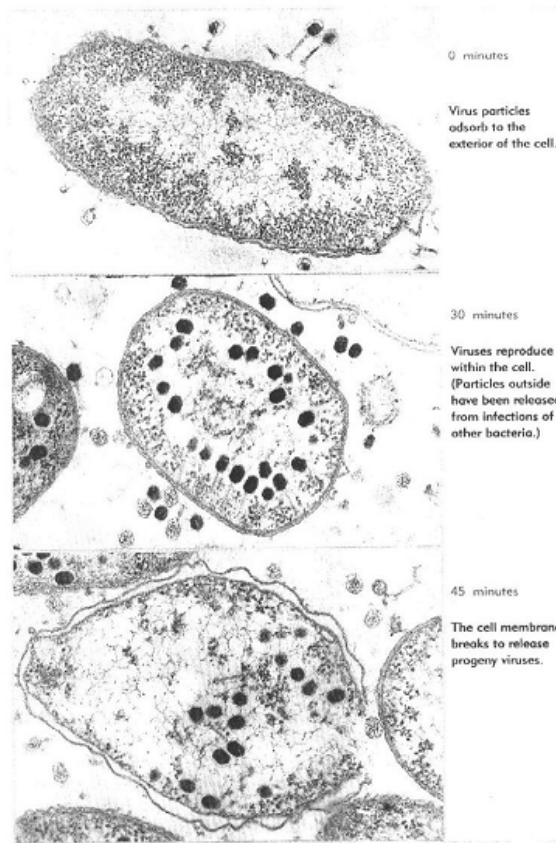
The time course of a phage infection. Phages and host bacterium are visualized by electron microscopy. Note the difference in size between the phage and the bacterium

Materials per team
(team size, 4 students per team)

- A tube with over-night culture of host bacterium, *Escherichia coli* BAU grown in LB-medium
- Phage preparation of. *E. coli* phage T4 (ca. 5 x 10^8 PFU per ml)
- 13 Petri dishes with LB-agar
- 13 Glass tubes with top agar (stored at 50°C)
- 13 Sterile glass tubes with lids
- 2 tubes with 9.9 ml LB-medium
- 2 tubes with 4.5 ml LB-medium
- 3 Glass flasks with 10 ml LB-medium

- Pipettes with sterile tips
- Incubator at 37°C
- Marker pen
- Timer

Practical work

Week 6.

1. Three 100-ml flasks, each holding 10 ml LB-medium, are marked A, B and C. Add 1 ml *E. coli* BAU over-night culture to flask A.

2. Incubate flasks A, B and C in a water incubator with shaking at 37°C for 45 min. Keep track of the time by a timer.

3. While the flasks are incubating you have 45 min to train the plaque assay. First prepare a dilution series of the phage stock. Be careful to take out the phage sample from the upper layer of the stock. The lower layer is chloroform used to conserve the phages.

   > **A reminder: How to make dilution series**
   > You should perform plaque assays for samples in the following dilutions: 10^{-5}, 10^{-6} and 10^{-7}. To obtain these dilutions:
   > First make a 100-fold (10^{-2}) dilution by transferring 0.1 ml of the phage stock to a tube with 9.9 ml LB-medium. Mark the tubes, mix carefully and shift the pipette tip between each dilution.
   > Next, make another 100-fold dilution to get to 10^{-4} by transferring 0.1 ml of the 10^{-2} dilution to a new tube with 9.9 ml LB-medium.
   > Then make a 10-fold dilution to get to 10^{-5} by transferring 0.5 ml of the 10^{-4} dilution to a tube with 4.5 ml LB-medium
   > Finally make two 10-fold dilutions to get to 10^{-6} and 10^{-7}.

4. Make plaque assays for the 10^{-5}, 10^{-6} and 10^{-7} dilutions. Make to assays one by one!
   For each assay;
   - First, transfer 0.1 ml of *E. coli* BAU culture (host bacterium) and 0.1 ml diluted phage sample to a sterile glass tube.
   - Then add 4 ml warm top-agar (tubes with 4 ml top-agar are in the incubator at ca. 50°C). Mix by rotating the tube between the palms of your hands and RAPIDLY pour the sample over a LB-agar plate labelled with the relevant dilution. Swirl gently to distribute the top layer and allow plate to harden.

5. When the agar plates have hardened they are placed at 37°C in an inverted position. After 1 - 2 days they are transferred to 4°C by the instructor.

6. When the *E. coli* BAU culture in flask A has incubated for 45 min the one step growth experiment is initiated.

   t = 0 min. Add 0.1 ml of the undiluted T4 phage stock. Again remember to take your phage sample from the top layer. Reset your timer. Return flask A to the incubator with shaking.

7. **t = 5 min**. Remove flask A from the incubator. Transfer 0.1 ml of the culture to flask B. Mix well and transfer 0.1 ml of the culture in flask B to flask C. We have now diluted the culture in flask A 10000 times. Return flask C to the incubator with shaking.

8. **t = 15 min.** Take one 0.1-ml sample and one 0.01 ml sample from flask C and transfer each sample into a sterile glass tube. Perform plaque assays for the two samples. The procedure is as in point 3 above: First mix your sample with 0.1 ml of the *E. coli* BAU culture. Next add 4 ml warm top agar and mix. Finally pour the sample over a LB-agar plate and swirl gently. Remember that top agar will solidify if kept too long at room temperature.

9. **t = 20, 25, 35 and 40 min.** Repeat procedure from t = 15 min.

10. When the agar plates have hardened they are placed at 37°C in an inverted position. After 1 - 2 days they are transferred to 4 °C by the instructor.

Week 7

1. Count all plaques in the range of 20 – 200 on each plate. Record your data in the below tables.

2. Calculate the number of plaque forming units (PFUs) per ml of the undiluted sample by the formula:
   Number of plaques x (sample dilution factor^{-1}) x (sample size in fraction of a ml^{-1})
   = plaque forming units (PFU)/ml.
   For example, 10 plaques in a 0.1 ml sample of a 10^{-5} dilution give 1 x 10^7 PFU ml^{-1}

Dilution series

Phage dilution	Number of plaques counted	PFU / ml of phage stock
10^{-5}		
10^{-6}		
10^{-7}		

One-step growth experiment

Time and sample size	Number of plaques counted	PFU / ml referring to flask C
15, 0.1 ml		
15, 0.01 ml		
20, 0.1 ml		
20, 0.01 ml		
25, 0.1 ml		
25, 0.01 ml		
35, 0.1 ml		
35, 0.01 ml		
40, 0.1 ml		
40, 0.01 ml		

Report

1. Present your data in the above tables.

2. Based on the data for the one step growth experiment, draw an infection curve (see figure 10.9 in the textbook).

3. Determine the latent period and the burst size for phage T4. Are your results in agreement with information available for T4 in the textbook or on the web?

4. You used a plaque assay to determine the number of T4 phages in your samples. However a plaque assay using *E. coli* BAU cannot detect all phages in a given sample. Explain why.

5. List other relevant methods whereby A) all viruses in a sample and B) other virulent bacterio-phages with other host bacteria could be detected and quantified.

6. Compare the impact of a lytic and a lysogenic phage infection on the fate of the host bacterium. Address possible harmful as well as beneficial effects of phage infection.

7. Mention the changes in a host bacterium that can lead to resistance to infection by a specific phage.

EXERCISE 9: BACTERIAL CONJUGATION

Introduction

Bacteria have three different mechanisms for genetic recombination that all involve direct or indirect transfer of DNA from a donor to a recipient. These are **transformation** (uptake of free DNA), **transduction** (transfer of bacterial DNA mediated by a bacteriophage) and **conjugation** (transfer of plasmid or chromosomal DNA from a donor bacterium to a recipient bacterium).

Conjugation is a mating process involving cell to cell contact between a donor and a recipient. The process results in uni-directional transfer of DNA from the donor to the recipient. By conjugation plasmid DNA can be transferred among bacteria. Conjugative plasmids carry genes, referred to as *tra* genes, which enable transfer. These genes take up at least 15 kb so conjugative plasmids are generally rather big. Some *tra* genes encode pili that are protein filaments whereby the connection between the donor and a recipient is established. Other *tra* genes are involved in replication of the plasmid DNA so that both the donor and the recipient each hold a copy of the plasmid when conjugation is completed. Recipients that have received a plasmid by conjugation are referred to as transconjugants.
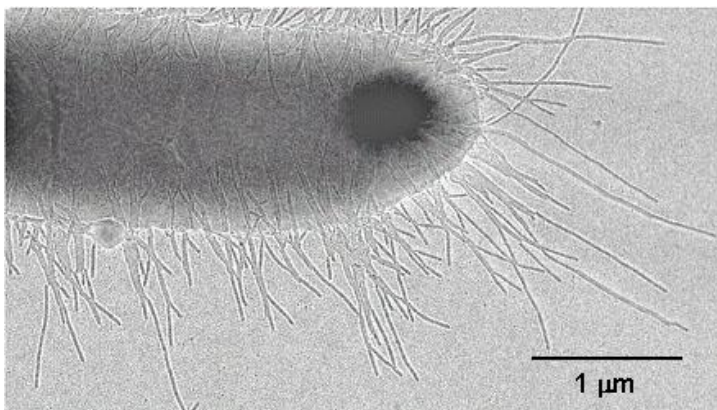
An *Escherichia coli* cell with pili extruding from the cell surface

Several conjugative plasmids are known. One important group is represented by resistance (R) plasmids carrying genes, which confer resistance to antibiotics and/or to heavy metals. Other conjugative plasmids carry genetic pathways involved in degradation of pesticides while still other plasmids encode restriction/modification systems conveying resistance to attack by bacteriophages.

The F factor or F plasmid (textbook, figure 11.19) is a conjugative plasmid that can also integrate into the host cell chromosome. The ability of the plasmid to insert into the chromosome is due to the presence of transposable elements on the plasmid. When identical or highly homologous transposons are present on the chromosome, the F-plasmid can be inserted by homologous recombination.

Cells possessing the F plasmid are referred to as F^+ and can donate the plasmid to F^- recipient cells during mating. If the F plasmid becomes integrated into the bacterial chromosome, the resulting cell is referred to an Hfr cell for high frequency of recombination, see textbook section 11.13. When an Hfr cell mates with an F^- cell, the plasmid transfer starts from a point in the plasmid DNA called origin of transfer (*oriT* in figure 11.23). However flanking chromosomal genes can also be transferred from donor to recipient, a process known a mobilization of chromosomal DNA. Chromosomal genes sitting right next to the F- plasmid are transferred early on during mating and with high efficacy, while genes further away from the inserted F- plasmid are transferred later on and with lower efficacy.

Aim of the exercise

The aims of this exercise are:
- to demonstrate the power of selective media for detection of gene transfer
- to demonstrate conjugative transfer of chromosomal genes from a donor to a recipient strain of *Escherichia coli*.
- to prepare a simple gene map

Experimental background and set up

In this exercise you will make a mating between a donor and a recipient bacterium. The donor is an Hfr prototrophic *E. coli* strain (that can make its own threonine, leucine, arginine, praline, histidine and praline for growth) that is streptomycin sensitive. The recipient is an F⁻ auxotrophic *E. coli* strain (that require threonine, leucine, arginine, proline, histidine and thiamine for growth) that is resistant to streptomycin.

The selective media used to obtain transconjugants contain streptomycin to eliminate growth of the Hfr donor. The growth of the recipient is prevented by the lack of essential growth factors. However the media contain arginine, histidine and thiamine as these are essential growth factors for the recipient, and will not be transferred by conjugation during the experiment.
The general outline of the experiments is found in the text below and on the figure on next page:

The experiment is initiated by mixing the Hfr donor and the recipient in a glass flask kept in at 37°C **without** agitation to keep the mating cells together. After 5 min the culture is diluted in order to make it less likely that novel conjugation pairs are formed. Hence, in the remaining part of the exercise we follow gene transfer initiated during the first 5 min.

The mating will be interrupted at three time points by mixing samples taken from the glass flask vigorously on a whirli-mixer as this treatment will break the pili holding donor and recipient together.

Finally transconjugants that have received genes for proline synthesis and/or for threonine/leucine synthesis will isolated on selective media.
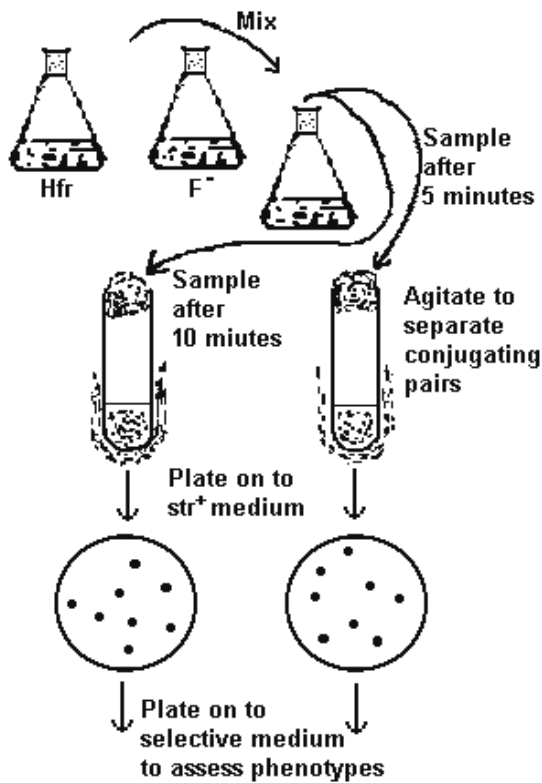
The general principle of an interrupted mating experiment. The sampling times on the figure does not apply to the present exercise.

Materials per team
(Team size: 4 students per team)

- 1 tube or flask with a growing culture of donor strain: *E. coli* W1034 (Hfr, str^S, pro$^+$, thr$^+$leu$^+$, arg$^+$, his$^+$thi$^+$)
- 1 tube or flask with a growing culture of recipient strain: *E. coli* W1022 (F$^-$, str^R, pro$^-$, thr$^-$leu$^-$, arg$^-$, his$^-$ thi$^-$)

- 3 Davis minimal A agar plates containing streptomycin (40 µg/ml), thiamine (0,5 ppm), histidine, arginine, threonine and leucine (50 ppm each) (DMA-agar).
- 3 Davis minimal B agar plates containing streptomycin, thiamine, histidine og arginine in concentrations as above (DMB-agar).
- 1 test tube containing 7 ml pre-warmed LB-medium – placed at 37⬚C.
- 1 sterile 100-ml glas flask
- Pipettes and sterile tips
- Eppendorph tubes with 0.9 ml P-buffer

- Water bath at 37°C
- Bunsen burner
- Drigalsky spatula and ethanol for sterilization

Practical work

Week 5

1. **t = 0 min**. Transfer 2.7 ml of the F⁻ culture (*E. coli* W1022) to a 100-ml glas flask. Then initiate the mating by adding 0.3 ml of the Hfr culture, W1034. Mix gently by swirling. Start your timer (**t= 0 min**) and place the flask in the water bath at 37°C **without** agitation.

2. **t = 5 min**. Add 7 ml of pre-warmed LB-medium to the mating mix. Thereby you dilute the culture and make it less likely that novel conjugation pairs are formed.

3. **t = 6 min**. Take out 0.1 ml of the mating mix and transfer the sample to a tube with 0.9 ml P-buffer. Agitate this sample vigorously my mixing on a whirli-mixer at highest speed for 30 sec to interrupt the mating.
   Immediately after, transfer 0.1 ml of the whirli-mixed sample to a DMA agar plate. Spread the inoculum over the agar plate by a sterilized Drigalsky spatula. Then transfer another 0.1 ml sample to a DMB agar plate and spread again as above.

4. **t = 20 min and t = 35 min.** Repeat the steps described in points 3 and 4 above.

5. Remember to mark your agar plates (not on the lid!) and incubate then in an inverted position at 37°C for two days. Then the teacher will transfer them to a cold room.

Week 6

1. Score the number of colonies appearing on the DMA and DMB agar plates. Calculate the number of transconjugants expressed as CFU / ml at 6, 20 and 35 min.

Report

1. Present your results as a table.

2. Explain how you have eliminated growth of donors and recipients on the DMA and DMB media you have used.

3. Describe the genotypes (combination of markers from donor and recipient) of transconjugants selected on DMA agar and on DMB-agar, respectively.

4. Which of these markers have been transferred first in your experiment? Does this result make sense when you compare it to figure 11.23 in the textbook?

5. Explain how conjugative chromosome mobilization and ordinary conjugation differ.

6. Discuss the significance of conjugation for the spread of antibiotics resistance in the environment.

EXERCISE 10: SELECTION OF BACTERIAL MUTANTS

Introduction

A mutation is an inherited change in base sequence of genome of an organism and, although mutations are rare, they represent an important source of genetic variability among living cells. In some cases the changes caused by a mutation enable the mutant cells to survive in a hostile environment. The type of mutation that confers some type of advantage to the mutant organism is referred to as a **selectable** mutation. Other mutations are non-selectable because they do not give a growth advantage to the mutant organism, although this type of mutations can result in very distinctive phenotypes.

An example of a selectable mutation is the development of antibiotic resistance. In an antibiotic-resistant bacterium the mutation might lead to a change in the permeability of the cell membrane so that access of the antibiotic to the cell is reduced. Alternatively the mutation could result in a change of the cellular target on which the antibiotic normally works, e.g., the cell wall or the ribosome. These mechanisms are generally different from the mechanisms behind plasmid-encoded resistance. Plasmid-borne genes will often encode enzymes that are able to degrade or modify the antibiotic or encode efflux pumps that can actively remove the antibiotic from the cell.

For comparison, an example of a non-selectable mutation is a mutation leading to auxotrophy. An auxotroph mutant is an organism that has lost the ability to synthesize an essential growth factor, for example an amino acid. The auxotroph mutant has a requirement for this growth factor, which must be included in the growth medium. The wild type organism from which the auxotroph was derived is called a prototroph.

The use of bacterial mutants continues to be a very powerful tool in bacterial genetics. For example the role of specific genes for important bacterial functions has been addressed through comparing the performance of mutant strains with that of the corresponding wild type. This procedure has been included when identifying the genetic background for production of bioactive metabolites, virulence factors, enzymes etc. Bacterial mutant strains are also important in test procedures for identifying hazardous chemicals in our environment, see for example the textbook section 11.7.

Consequently it is important to have simple methods whereby mutants can be detected and isolated. A selectable mutant can be isolated by direct selection. Direct selection uses selective media supporting growth of the mutant while the parent wild type will be eliminated. Some non-selectable nutritional mutants can be isolated by indirect selection using the replica plating method, see below.

Aim of the exercise

The aims of this exercise are:
- to isolate steptomycin-resistant mutants in a sensitive bacterial population by direct selection;
- to isolate leucine-auxotroph mutants in a prototrophic bacterial population by indirect selection using the replica plating technique.

Isolation of a streptomycin-resistant mutant

Experimental background and set-up

The experiment permits you to isolate a streptomycin-resistant mutant from a streptomycin-sensitive *Escherichia coli* culture. You will use the gradient-plate technique illustrated in the figure below. In brief, a gradient plate is prepared by pouring a lower slanted layer of agar medium without streptomycin. When this layer has solidified, another layer of agar medium, this time containing streptomycin, is poured on top. The complete plate will contain a concentration gradient of streptomycin. When you inoculate the plates by spreading a liquid culture on top of the plate, streptomycin-resistant colonies emerge in the region of the plate with a high streptomycin concentration

The figure shows a very simple representation of the principle behind a streptomycin gradient plate. The white layer represent agar without streptomycin, and the black agar with the antibiotic included

Materials per team
(Team size: 4 students per team)

- 1 tube or flask with an over-night culture of *Escherichia coli* DSM 498 grown in LB-medium.
- 2 gradient plates prepared from 10 ml LB-agar + 10 ml LB agar containing 0.03 mg streptomycin per ml. The high concentration area is marked with a black dot.
- Pipettes and sterile tips
- Bunsen burner and ethanol for sterilization
- Drigalsky spatula
- Incubation loop

Practical work

Week 5

1. Add 0.2 ml of the over-night culture of *E. coli* to the gradient plate. Spread the culture over the surface of the plate by a sterilized Drigalsky spatula.

2. Incubate the plate (bottom up) at 37°C for 48 h. The teacher will transfer the plates to a cold room until next week.

Week 6

1. Select a bacterial colony from the middle of the plate and another from the region with high streptomycin concentration.

2. With a sterile inoculation loop, streak the two selected colonies, one after the other, from the low concentration to the high concentration region of a new gradient plate.

3. Incubate the plate (bottom up) at 37°C for 48 h. The teacher will transfer the plates to a cold room until next week.

Week 7

1. Measure the lines of growth for the two selected isolates. Can they grow at high streptomycin concentrations?
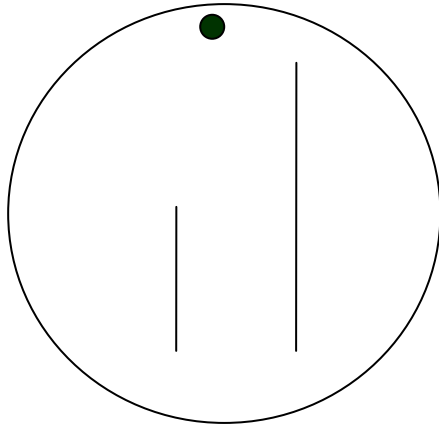

Example of how results from the gradient plate experiment can be presented. The black dot indicates the high streptomycin concentration area of the plate

Demonstration of a leucine-auxotroph mutant by replica plating

Experimental background and set-up

In this part of the exercise we will look for leucine-auxotrophs in a prototroph wild-type population of the bacterium *Serratia marcescens*. In brief, mutant and wild-type bacteria are cultured on L-agar (a supplemented medium) prior to the experiment. The L-agar plate is used as a master plate, see below figure. A replica of the colonies growing on the master plate is made on a velvet surface and transferred to a replica plate containing a minimal medium without leucine. Subsequently a second replica is made to a minimal medium with leucine (supplemented medium) to check to transfer efficacy of the experiment. This is not shown on the below figure. The plates are incubated to allow for colony development, and colonies are scored on all plates.
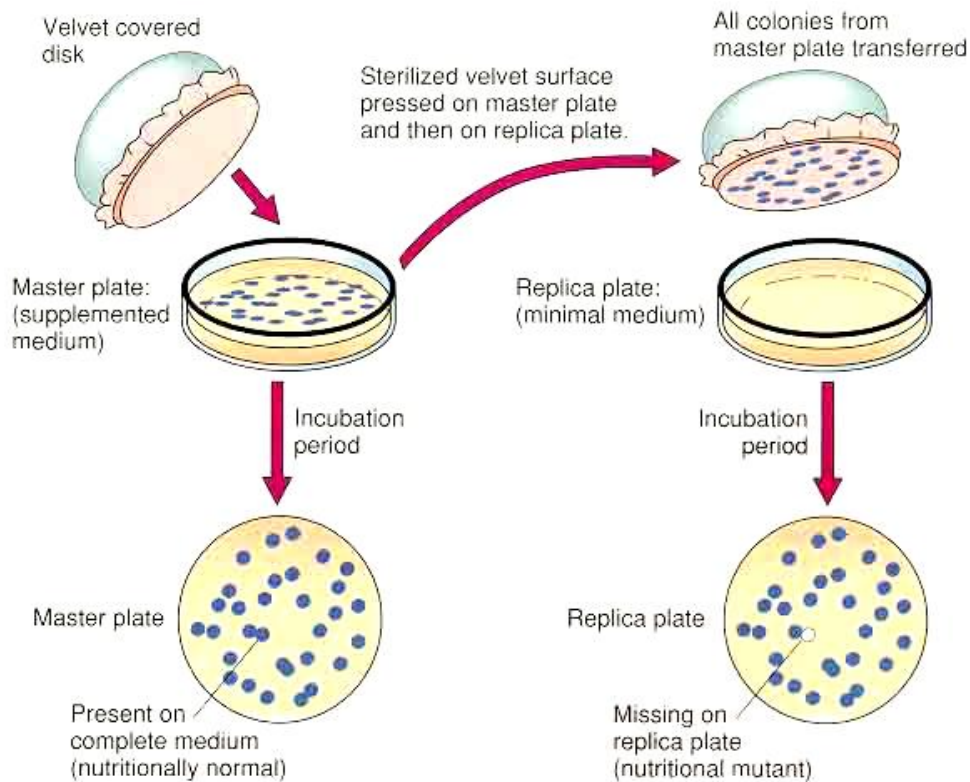
Principle of the replica plating technique used to isolate a nutritional mutant

Materials per team
(Team size: 4 students per team)

- 1 LB-agar plate with 20-40 colonies of *S. marcescens* grown at 37°C
- 1 agar plate with Davis minimal agar
- 1 agar plate with Davis minimal agar with leucine
- 1 Sterile sheet of velvet
- 1 rubber stopper to be used for the transfer
- 1 ink marker

Practical work

Week 6

1. Mark the master plate, the rubber stopper and the two replica plates by the ink marker so that they can be all be oriented relative to each other.

2. Place stopper on the table and suspend the sterile velvet sheet on top of the smaller side on the stopper – the soft velvet side should face up. Only handle the sheet at the margins to avoid contamination of the areas used for transfer of bacterial colonies.

3. Remove the lid of the master plate and place the agar surface on top of the velvet. Gently press the agar plate downwards until you can see that the colonies make contact with the velvet.

4. Transfer the replica to the agar plate with minimal medium and thereafter to the minimal medium

45

with leucine. When you make the replicas you should gently press the replica plate down towards the velvet until you can see that the colonies on the velvet contact the agar surface. Remember keep the same orientation of the master plate, the stopper and the replica plates. After the two transfers, the velvet used for the transfer should be soaked in disinfectant. Ask the instructor.

5. Incubate the plates at 37°C for 1 – 2 days. They will then be transferred to a cold room by the teacher.

Week 7

1. Count the number of colonies on the master and on the two replica plates.

Report

1. Present the length of the growth lines on the two selected "streptomycin-resistant" isolates and comment whether you consider them as streptomycin resistant

2. Explain the principle behind a direct selection procedure and give examples of other types of mutants that can be isolated after direct selection.

3. In recent decades there has been an increase in drug-resistant bacteria in the environment. Please explain which type of mutations that could contribute to this increase.

4. Is the occurrence of streptomycin-resistant bacteria in your experiment affected by the presence of streptomycin in the gradient plates?

5. Present the colony counts from the master and replica plates. How many auxotrophs and how many prototrophs did you find? Has the transfer from master to replica plates been efficient?

6. Define an auxotroph versus a prototroph bacterium. Why can't we isolate an auxotroph mutant by direct selection?

CULTURES, MEDIA AND REAGENTS

EXERCISE 1

High-agar medium in Petri plates
20 g agar/l

EXERCISE 2

Cultures
Cultures of four different bacteria will be examined in the exercise. The identity of the bacteria will be revealed at the end of the exercise

Liquid LB medium (L-bouillon)
Tryptone 10 g/l
Yeast extract 5 g/l
NaCl 10 g/l
Glucose 1g/l

Solid LB medium (L-agar)
Liquid LB medium solidified in 15 g agar/l

Methylene blue
0.3% in alkaline ethanol-water (0.3 g methylene blue dissolved in 30 ml 96% ethanol and 100 ml 0.01% KOH)

Crystal violet
1% in water

J-KJ (iodine, Lugols)
4% in water (4 g J and 3 g KJ in 100 ml water)

Ethanol
96%

Safranine
1% in water

EXERCISE 3

H_2O_2, hydrogenperoxide
3% in water

Tetramethyl-p-phenyldiamine hydrochloride
1% in water (use dark bottle and add 0.1% ascorbic acid to avoid auto-oxidation)

Solid casein medium (skim milk agar)
Skim milk 300 ml/l
Agar 15 g/l

Semi-solid Hugh-Leifson (H-L) medium
$(NH_4)_2HPO_4$ 0.4 g/l
K_2HPO_4 0.3 g/l
NaCl 5 g/l
Glucose 10 g/l
Agar 3 g/l
Bromothymol blue 3 ml/l of 1% solution in water (add after agar is melted)

EXERCISE 4

Cultures
Cultures of four different bacteria will be examined in the exercise. The identity of the bacteria will be revealed at the end of the exercise

Mineral oil

TDA reagent
$FeCl_3$ 34 g/l

JAMES reagent
Compound J2183 (confidential) 5 g/l of 1 N HCl

VP1 reagent
KOH 400 g/l

VP2 reagent
Alfa-naphtol 60 g/l of 96% ethanol

NIT1 reagent
Sulphanilic acid 4 g/l of 30% acetic acid

NIT2 reagent
N,N-dimethyl-naphthylamine 6 g/l of 30% acetic acid

Zn powder

EXERCISE 5

Cultures
Culture 1: *Serratia marcescens.* Culture 2: *Pseudomonas fluorescens*

The bacterial strains are kept at -80°C. The frozen cultures are transferred to solid LB and will be ready for growth experiments in liquid media the following day.
At 1 to 5 h interval, flasks with ml LB media are inoculated with 50 µl of a dense and exponentially growing culture of each bacterium.

LB-medium (Luria-Bertani) broth
Tryptone   10 g/l
Yeast extract   5 g/l
NaCl   10 g/l

Petri dishes with LB (Luria-Bertani) agar
To liquid LB media is added 1.5% agar (= 15 g/l).
Add 20 ml warm (>50°C) LB agar to each plate. When stiff (about 1 h), the plates are ready for cultivation.

PBS (Phosphate Buffered Saline) buffer
NaCl   32 g
Na_2HPO_4 4.6 g
KCl   0.8 g
KH_2PO_4 0.8 g
Water   4 liters
The pH should be between 7.28 and 7.60, otherwise remake the solution.

SYBR Gree1 solution
15 µl SYBR Green1 stock (Molecular Probes, product No. S-7563) diluted in 1 ml water.

EXERCISE 6

Cultures
Exponentially growing cultures the following cultures:
Pseudomonas sp.
Bacillus stearothermophilus
Escherichia coli
P. fluorescens
S. marcescens
Alicyclobacillus acidocaldarius

LB-medium (Luria-Bertani) broth
Tryptone   10 g/l
Yeast extract   5 g/l
NaCl   10 g/l
pH and salinities of the media will be adjusted by addition of 1 M HCl, 1 M NaOH or NaCl.

EXERCISE 7

Cultures
An exponentially growing culture of *Serratia marcescens* strain W225. Media: Liquid LB media.

LB-medium (Luria-Bertania) broth
Tryptone   10 g/l
Yeast extract   5 g/l
NaCl   10 g/l

LB plates (Petri dishes)

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agar	15 g/l

Dilution media

0.9% NaCl: 9 g NaCl to 1 liter of water

EXERCISE 8

Cultures

Escherichia coli strain BAU. The strain is routinely cultured on LB-agar. Cultures in LB-medium is inoculated the day before the exercise and incubated at 37 °C with agitation over-night.

Phage T4 suspended in LB-medium conserved by 1 drop of chloroform per ml. The phage stock is stored at 4 °C.

LB-medium (Luria-Bertani medium)

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

pH is adjusted to 7.0 ± 0.2 with NaOH.
The medium is autoclaved at 121 °C for 20 min.

LB-agar

The above LB-medium is solidified by the addition of 15 g agar/l

Top-agar

The above LB-medium is solidified by the addition of 10 g agar/l

EXERCISE 9

Cultures

Donor strain: *E. coli* W1034 (Hfr, str^S, pro$^+$, thr$^+$leu$^+$, arg$^+$, his$^+$thi$^+$)
Recipient strain: *E. coli* W1022 (F$^-$, str^R, pro$^-$, thr$^-$leu$^-$, arg$^-$, his$^-$thi$^-$)

To instructor: Growing cultures of donor and recipient are prepared by diluting over-night cultures grown in LB-medium to OD_{600} = 0.1. Use LB-medium for the dilution and then grow 50 ml of the diluted cultures in 300 ml-flasks at 37°C without agitation for about 3 hours until they reach an OD_{600} of about 1.0. These cultures contain about 2×10^8 cells per ml

Davis minimal A agar

10.6 g/l Davis Minimal Broth (Difco), 4 g/l glucose, 15 g/l agar and supplemented with streptomycin (40 µg/ml), thiamine (0,5 ppm), histidine, arginine, threonine and leucine (50 ppm each) (DMA-agar).

Davis minimal B agar

10.6 g/l Davis Minimal Broth (Difco), 4 g/l glucose, 15 g/l agar containing streptomycin, thiamine, histidine og arginine in concentrations as above (DMB-agar).

P-buffer
Solution 1: 7 g/l Na_2HPO_4, $2H_2O$; 3 g/l KH_2PO_4, 4 g/l NaCl.
Solution 2: 0.25 g/l $MgSO_4$, $7H_2O$
Autoclave separately, then mix 1000 ml of solution 1 with 2 ml of solution 2.

Exercise 10

Cultures

Escherichia coli DSM 498, overnight culture in LB-medium.
S. marcescens strain W225 (wt)
S. marcescens strain W315 (*leu*-)
These two *S. marcescens* strains are stab inoculated on LB-agar plates in a proportion, which varies from master plate to master plate. Plates are incubated at 37°C.

Gradient plates prepared from 10 ml LB-agar + 10 ml LB agar containing 0.1 mg streptomycin per ml. The high concentration area is marked with a black dot.

Davis minimal agar
Davis Minimal Broth (Difco)   10.6 g/l
Agar   15 g/l
Autoclave at 121°C for 15 min
Add 10 ml 40 % glucose (Sterile Filtered) after autoclaving (final conc. 4 g/l)

Davis minimal agar with leucine
Add leucin 50 mg/l to the above

Gradient plates
Are prepared from 10 ml LB-agar + 10 ml LB agar containing 0.1 mg streptomycin per ml.
LB-medium (Luria-Bertani medium)
Tryptone   10 g/l
Yeast extract   5 g/l
NaCl   10 g/l
pH is adjusted to 7.0 ± 0.2 with NaOH.
The medium is autoclaved at 121 °C for 20 min.

LB-agar
The above LB-medium is solidified by the addition of 15 g agar/l