Institute of Microbiology

ETH Zürich

P GL der Biologie I

MICROBIOLOGY

FS2017 Edition

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Contents

Recommended Literature

"Allgemeine Mikrobiologie" by Georg Fuchs und Hans G. Schlegel, Thieme, 9. Auflage 2014
"Taschenlehrbuch Biologie: Mikrobiologie" by Katharina Munk, Thieme, 2008
"Brock Mikrobiologie kompakt" by Michael T. Madigan, John M. Martinko, David A. Stahl, David P. Clark, Pearson, 13. Auflage 2015

Recommended websites

http://tolweb.org/fungi
http://tolweb.org/archaea
http://tolweb.org/eubacteria

1. BASIC TECHNIQUES AND TOOLS FOR THE WORK WITH MICROORGANISMS

Introduction

Use of adjustable micropipettes

Adjustable micropipettes for dispensing small volumes of liquid have become an important tool in molecular biology. They have to be handled with great care. Their correct use is explained below. In this course, you will be using a set of three micropipettes: Gilson P1000, P200 and P20 (see Fig. 1.1). The numbers stand for the UPPER LIMIT of the volumes (in µl) that can be pipetted with these pipettes. Lower volumes can be pipetted by adjusting the volume of choice (indicated in the pipette window) using the black screw on the top of the pipette. PLEASE do not adjust the volume below the LOWER LIMIT of the pipettes because (1) you will damage the pipette (each costs around SFr. 250.-) and (2) your pipetting will not be accurate. The respective lower volume limits of the pipettes are:

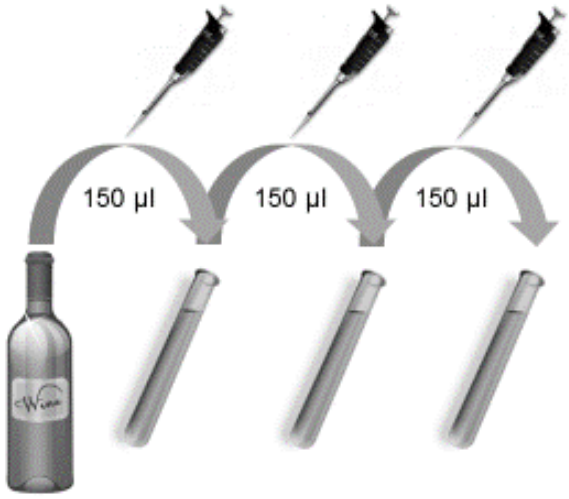
P1000: 100 µl
P200: 20 µl
P20: 1 µl

Use the BLUE TIPS for the P1000 and the WHITE TIPS for the P200 and P20. For pipetting attach the appropriate tip, press the stamp carefully TO the first pressure point, place the tip into the solution, SLOWLY release the stamp and monitor the uptake of the liquid by eye, take the pipette out of the solution and place it into the recipient container, release the liquid by pressing the stamp BEYOND the first pressure point, take the pipette out of the recipient container, release the stamp and eject the tip using the white button on the small white button on the top of the pipette. Now you are ready for a new round of pipetting.

Figure 1.1: Gilson Micropipette

Preparation of dilution series

The preparation of dilution series is used frequently during this course and often causes problems. For this reason this technique is briefly illustrated below:



Volume (before dilution)	x µl	1350 µl	1350 µl	1350 µl
Dilution	1fold	10fold	100fold	1000fold
Concentration (after dilution)	1	0.1	0.01	0.001

For the preparation of a series of 10fold dilutions of a sample of a given concentration (here: 1), tubes containing 9 volume units (VU; here: µl) of a diluent (e.g. water) are prepared and labelled. Subsequently, 1 VU of the sample to be diluted is pipetted into the first tube and mixed by pipetting and vortexing. Using the same or a new pipette tip, 1 VU of the 10fold dilution is pipetted into the second tube and the resulting 100fold dilution is mixed again. Further dilutions are prepared likewise. NOTE: All tubes with the exception of the last one contain only 9 UV. The reduced volumes have no influence on the concentrations.

Culture media and growth conditions

The composition of culture media has to be adapted to the requirements of the microorganisms which are going to be cultured. Many bacteria are undemanding and will grow in media of simple composition, while other bacteria depend on the presence of certain trace elements or vitamins. Standard media for the routine cultivation of microorganisms for clinical diagnostics, food industry or research laboratories are commercially available.

The cultivation is done either on solid or in liquid culture media. For the preparation of solid media 1.5-2 % of agar is added to the medium. Agar is a polysaccharide which is extracted from algae and provides a gel-like consistency to the medium. Agar melts at 100°C (212°F) and solidifies only at a temperature of around 45°C (113°F). Agar is, with very few exceptions, not used as a nutrient source by the microorganisms.

The most common cultivation containers for agar cultures are petri dishes or test tubes with a slope agar layer. Bacteria in liquid cultures are cultivated in test tubes or in Erlenmeyer flasks. Bigger cultivation containers like fermenters or bioreactors are used for the production of larger amounts of microorganisms and isolation of their metabolites.

Oxygen is essential for the cultivation of most bacteria. There are, however, anaerobic bacteria growing only in the absence of oxygen. Anaerobic conditions are achieved by binding the oxygen to an oxygen-absorbing agent (e.g. alkaline pyrogallol) or by cultivation in chambers or containers with a defined atmosphere, containing mainly nitrogen (see Experiment 6.2).

The cultivation and incubation temperature for most microorganisms is between 20°C and 37°C (68 – 95°F).

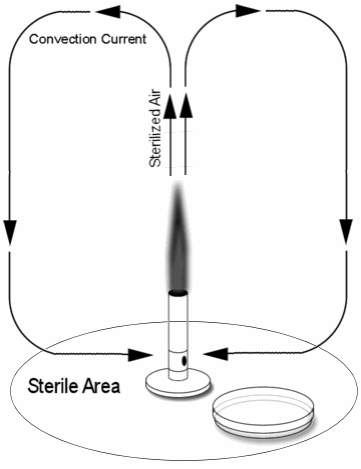
Sterilisation

To obtain reproducible results with bacteria or fungi, pure cultures are required. Maintaining pure cultures again calls for sterile cultivation containers, culture media and instruments. Below, different sterilisation methods are described.

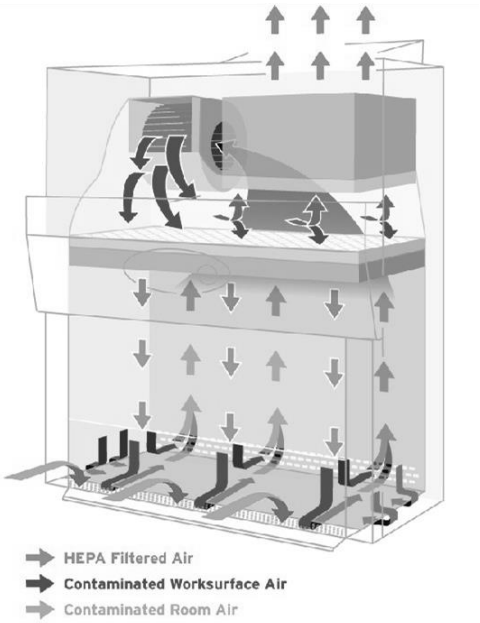
Sterilisation by dry heat

Annealing of inoculation tools: This sterilisation method is used for metal. It is a fast method, mainly used for the sterilisation of inoculating loops. The annealing is done by holding the inoculating loop into the oxidising flame of the Bunsen burner until it is glowing. For inoculating needles and loops, also the metallic part of the fastener has to be heated. It is important to let the inoculating loop cool down prior to contact with microorganisms. The sterilisation of these instruments has to be done also immediately after use, in order to prevent contamination of the surrounding area.

Flaming: Flaming is mainly used for the outside sterilisation of glass and metal containers in order to sterilize their seams. In addition, the air convection which results from a flame, prevents the intrusion of germs in the immediate surrounding of the flame:



This technique of creating a sterile area is sufficient for most experiments in microbiology and will also be used in this course. Sterile hoods where the working area is continuously flushed with sterile-filtered air are required for experiments that are more susceptible for contamination, e.g. inoculation of slow growing microorganisms, or handling of pathogenic microorganisms:



Hot air sterilisation at 160°C (320 °F) for 2 hours in the drying oven: Suitable for empty glass containers, glass pipettes and surgical instruments.

Sterilisation by damp heat

Damp heat (saturated steam) is much more effective than dry heat. Thermostable liquids (e.g. many culture media) are sterilised this way.

Autoclaving: In an autoclave the sterilisation is performed with pressurized steam (2 bar, 29 psi) at a temperature of 121°C (250°F). In order to achieve a pure vapour phase, the air must be replaced by the exhaustion of steam from the autoclave. The actual sterilisation follows afterwards for 20 minutes at 121°C.

Sterilisation by filtration

Liquids which contain heat sensitive substances are sterilised by filtration. Filters which have to hold back bacteria, should have a maximal pore size of 0.45 µm. Sterile disposable filters are available with pore sizes from 0.2 to 5µm. Filters with a small pore size reduce the flow rate. This effect can be reduced by applying pressure or vacuum (see Experiments 2.6 and 2.4, respectively).

Sterilisation by fumigation

Heat sensitive materials made of plastic (petri dishes, syringes, filters) and disposable material made of glass (pipettes) are sterilised with ethylene oxide before being shrink-wrapped. Sterile disposable plastic material used in our institute is purchased.

Sterilisation by irradiation

After the usage of ionising radiation, especially for the sterilisation of food, had become less important, this method has again gained in importance because of bioterrorism. Presently all mail that is sent to

official institutions in the USA is irradiated in order to kill pathogen germs, such as *Bacillus anthracis*, the causative organism for splenic fever (anthrax).

Inoculation techniques

While inoculating liquid or solid media with bacteria, special attention must be paid avoiding contamination with impurities or external germs. The introduction to the most common inoculation techniques is part of the following experiments including Chapters 1 and 2.

Experimental Part

Experiment 1.1: Preparation of a liquid culture medium (nutrient broth)

As mentioned above, the cultivation of microorganisms requires sterile culture media which can be either liquid or solid. The following procedure is therefore daily routine in a microbiology lab. In a first experiment, 400 ml liquid Luria Bertani Glucose (LBG) broth is produced for cultivation of the bacterium *Escherichia coli* in Experiment 6.1 as part of Courseday 3.

Procedure:

Weigh the respective amounts of the various components of the medium (see Addendum – leave away Agar for liquid media!) on a balance (since these are fine powders: use spoon to protect your lungs!) and transfer to a graduated 500 ml cylinder containing 100 ml of deionized water. Add deionized water to 400 ml and dissolve the medium using a magnetic stir bar. Transfer the solution to a 500 ml-Schottflask, mark flask with autoclaving tape and label with 'Practical course E394', group name and the date. Place the flask in a dedicated metal container in the autoclaving room.

Experiment 1.2: Isolation of single colonies for the production of pure cultures

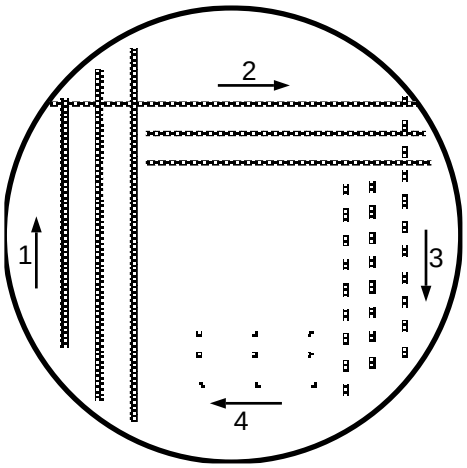
The isolation of single colonies and the production of pure cultures starting from a mixed culture is an essential issue in Microbiology. A simple and ingenious method developed by Robert Koch is based on the growth of microorganisms on solid media. A single colony (clone) results from the multiple divisions of a single bacterial cell and thus represents a pure culture. In order to obtain single colonies a bacterial suspension has to be diluted and plated. Various dilution and plating methods can be used. In this experiment, dilution streaking is used for the isolation of single colonies and, thus, pure cultures from a mixture of two different bacteria, *Escherichia coli* and *Serratia marcescens*. Alternative dilution and plating methods will be used in to assess the number of cells in a yeast colony (Experiment 4.1) or in environmental soil and water samples (Chapter 2).

Characteristics of used bacteria:

Escherichia coli is a Gram-negative, facultative anaerobic, peritrichous rod. It is part of our natural intestinal flora. *E. coli* is also able to survive outside the intestine and can easily be detected. Therefore it is very important for the assessment of the water quality. The presence of *E. coli* in water samples indicates a faecal contamination. The so-called “coli-number” indicates how many reproducing cells of *E. coli* are contained in one millilitre of sample water. According to the Swiss food

regulation (Lebensmittelverordnung LMV) no coli bacteria or coliform bacteria are allowed to be detected in 100 ml of untreated drinking water. For the detection of the coli number, special indicator media (e.g. MacConkey-agar or TBX-agar, see addendum) are used on which *E.coli* and other coliforms show a characteristic colouring which allows a first differentiation (see Experiment 2.4). Special, well defined laboratory strains of *E. coli* are used for genetic engineering.

Serratia marcescens is a Gram-negative, facultative anaerobic, motile rod. It forms a deep red pigment, prodigiosin. The backbone of this pigment consists of three pyrrol rings. In the past *S. marcescens* was called 'housel fungus' ('bloody bread'), because of its ability to grow as a red culture on moist bread.



Procedure:

30 µl of a mixed culture is streaked out with an inoculating loop in 2 to 3 parallel strokes on the agar medium in a petri dish (1). The loop is then flamed and the petri dish is turned by 90°. Originating from one end of the parallel strokes bacteria material is again streaked out in a 90° angle. If this procedure is repeated one or two times, it should be possible to obtain single colonies after 24-48 h incubation.

Results:

Indicate the number of obtained white or red single colonies in below table:

	White (*E. coli*)	Red (*S. marcescens*)
# of single colonies		

Personal notes:

2. ISOLATION OF MICROORGANISMS FROM THE ENVIRONMENT

Introduction

Microorganisms are found everywhere, around us, on us and even within us, but they are so small that we are usually not aware of them. In this chapter, you will learn some methods to sample and visualize these microorganisms. Most of these methods are based on cultivation of the microorganisms on solid culture media and evaluation of the macroscopically visible, microbial colonies. The number of these colonies or Colony Forming Units (CFUs) is a reflection of the abundance of bacterial cells and fungal spores in the original sample as a colony usually originates from a single bacterial cell or fungal spore. One should be aware, however, that this number is critically depending on the cultivation conditions used. Most environmental microrganisms (probably > 90%) cannot be cultivated under commonly used culture conditions.

Microorganisms on surfaces

Experiment 2.1: Sampling of microorganisms using Rodac plates

With this technique bacteria on surfaces are detected. This method is important in food hygiene and e.g. used to examine surfaces in restaurant kitchens or in food producing factories for the presence of health-endangering microorganisms. Rodac plates are similar to petri dishes but they are furnished with a double seam (see Fig. 2.1). The plate is filled with a LBG agar cushion.

Figure 2.1: Rodac plate. Petri dish with double seam to sample microorganisms on surfaces.

Material: 1 Rodac plate per student

Procedure:

The agar cushion in the Rodac plate is pressed lightly onto the surface to be checked for 10 seconds and is afterwards incubated for three days at 30 °C. This way, a “finger print” of the examined surface is obtained. Surfaces to be considered for examination: hands (both washed and unwashed - see title page of the script), surfaces on the body, towels, etc.

NOTE: Metal surfaces are usually very clean in microbiological terms due to toxic metal ions (Ag, Ni, Cu) present in many metal alloys.

Results:

Surface	Number of bacterial colonies	Number of fungal colonies

Microorganisms in the air

Air also contains many microorganisms e.g. spores of fungi and bacteria. Analysis of microorganisms in the air is very complex because of the large fluctuations in the counts of microorganisms, which is illustrated in the following table.

Examples of microbial counts in the air in different locations:

Location	Number of samples	Microorganisms/m^3 average	Microorganisms/m^3 extremes
Arosa (open field)	12	130	24/220
Klotener Ried (open field)	18	218	64/343
Arosa (village border)	12	244	83/458
Kloten (crossroad)	24	398	134/1236

There are different methods for the isolation of microorganisms in the air. The simplest one is to expose agar plates for a certain time to allow bacteria and fungi to sediment. Colonies are counted after an incubation period of 24-48 h at room temperature. This method will be used in Experiment 2.2. A more reliable method is the use of automatic air samplers. With this method, a defined amount of air is aspirated and thrown onto a medium on which the microorganisms stick.

NOTE: The selection of the medium is very important for these and any cultivation-dependent method of microbe detection (see Addendum for recipes of some selected media). For bacteria, Plate Count (PC) or Luria Broth Glucose (LBG) agar are suited. For the selective cultivation of fungi Malt Agar (MA), Potato Dextrose Agar (PDA) or Sabouraud-Dextrose agar (SD) are used. In this course, LBG and MA agar plates are available.

Experiment 2.2: Microbial air analysis using spontaneous sedimentation

Material:

1 LBG and 1 MA agar plate per student

Procedure:

Open agar plates are exposed in different locations for 60 minutes. Afterwards the plates are closed and incubated at room temperature for 24-48 h. As previously mentioned, the LBG agar promotes the growth of bacteria, the MA agar the growth of fungi.

Results:

Medium	**Location**	**Number of bacterial colonies**	**Number of fungal colonies**
LBG			
MA			

Microorganisms in the soil

Soil is very rich in microorganisms including bacteria and fungi. These organisms are responsible for recycling the plant-derived organic material by degradation and finally mineralization (see also Experiment 6.6). The number and species of soil microorganisms is subject to a great dynamic and diversity which depends on climatic conditions and characteristics of soils. One of the best-known soil borne bacteria are the globally omnipresent spore formers belonging to the genus *Bacillus* (Family: Bacillaceae). The rod-shaped bacteria are gram-positive and grow under aerobic conditions. They start to form endospores (see Fig. 2.2) as soon as one of the essential nutrients for vegetative growth is depleted. The spores are very tolerant against environmental impacts including high temperatures, and able to survive for years. The spores germinate again if growth conditions become favorable. Many *Bacillus* species produce biologically active secondary metabolites. For example, *Bacillus thuringiensis* (Bt) produces during early sporulation proteins which are highly toxic against larval stages of invertebrates. The proteins are deposited within the cells as separate bodies, still visible in the light microscope. In many cases these proteins are assembled to crystals. Upon ingestion by target insects the crystals are dissolved and the proteins undergo activation by gut juice proteases. The resulting toxin destroys the gut epithelium. The larvae are killed. The Bt spores can germinate and proliferate in the dead insect body. This feeding style of microorganisms is called necrotrophy. Other examples of spore-forming soil bacteria are actinobacteria, also referred to as streptomycetes, which form multicellular filaments and are famous for their ability to produce antibiotics (see Chapters 3 and 5).

In the following experiment, the ratio between the total number of heat-resistant vs. heat-sensitive aerobic, mesophilic bacteria in different soil samples will be determined. In addition, the enrichment of endospore-forming *Bacillus* sp. by heating of the bacterial suspension will be verified by microscopy.

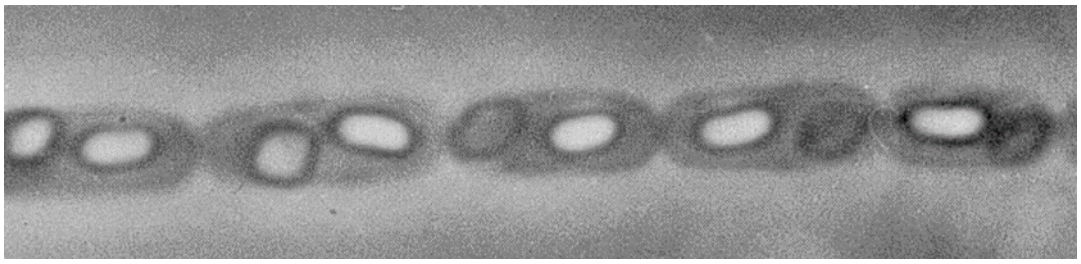


Figure 2.2: Micrograph of Gram-stained *Bacillus sp.* cells. Dark rods: vegetative cells; white ellipsoids: endospores; dark rhomboids: toxin crystals. *Picture provided by P. Lüthy*

Experiment 2.3: Isolation of soilborne bacteria and enrichment of spore-forming bacteria

Material:

- Soil samples (own, forest, meadow, field)
- 90°C-Heizblock
- 6 LBG-agar plates per group of students

Procedure:

- Suspend 2-3 spatula tips of soil in approximately 10 ml of sterile water in a sterile 15 ml Falcon tube (vortex).
- Let larger particles sediment.
- Transfer 1 ml of the supernatant to a sterile Eppendorf tube.
- Prepare a 3-step dilution series (10^{-1}, 10^{-2}, and 10^{-3}) in 1 ml of sterile water.
- Plate 250 µl of each of the diluted sample on LBG using the surface plating method (see pp. 13).
- Incubate the rest of the dilutions for 10 min at 90°C.
- Plate 250 µl of the heated dilutions on LBG using the surface plating method.
- Incubate all plates at RT.

Results:

1) Record the number of bacterial colonies as means of the number of viable bacterial cells in the samples:

Source / type of soil	# of bacteria/ml in non-heated sample	# of bacteria/ml in heated sample

NOTE: Count colonies on dilution with countable number of colonies and calculate the number of colonies per ml by considering the dilution factor and the volume plated.

2) Check colonies which have survived the heat treatment for the presence of endospores. Under the microscope in the phase contrast mode bacterial spores appear as bright cells (see Fig. 2.2 and instructions for the microscopy of microorganisms on pp. 24).

Microorganisms in water

Most liquids harbour a natural microflora. The quality of drinking water (including beverages) is therefore checked very strictly by the authorities based on the regulations in the “Schweizerisches Lebensmittelbuch” and the "Hygieneverordnung des EDI (SR817.024.1). Indicator bacteria are aerobic mesophilic microorganisms, *E. coli*, Enterococci and *Pseudomonas aeruginosa*. Some threshold values of drinking water are given in the table below.

Drinking water	**Aerobic, mesophilic bacteria (CFUs)**	***E. coli* and Enterococci (in 100 ml)**	***Pseudomonas aeruginosa* (in 100 ml)**
at catchment	100/ml	not detectable	not detectable
in the water lines	300/ml	not detectable	not detectable
mineral water bottled	20/ml	not detectable	not detectable

CFU: Colony forming unit

Raw water from a sewage treatment plant contains a high concentration of enteric bacteria of human origin e.g. *E. coli*. This high concentration of a rather specific group of bacteria is exploited for two purposes in this course:
(1) **Determination of the abundance of *E. coli* among enteric Gram-negative bacteria.**
(2) **Detection of *E. coli*-specific bacteriophages.** Large populations of bacteria in the environment are often accompanied by the respective bacteriophages. Bacteriophages are viruses that have specialized for bacteria as hosts (see Experiment 2.6). Since bacteria have a diameter from 0.8 to 3 µm and bacteriophages a diameter of 0.01 to 0.100 µm, they can be separated from each other by filtration. Upon isolation, the phages can be tested for the presence of a host bacterium of interest e.g. *E. coli*.

For the inoculation (plating) of microorganisms in liquid samples, different methods can be used depending on the volume of the sample. For smaller volumes, either the plate pouring or surface plating method are chosen. For larger volumes, usually the membrane filter technique is used:

Plate pouring method: As already described above (Experiment 2.3.) the microbial sample (e.g. 1 ml) placed in a petri dish and carefully mixed with liquid agar medium by circular movements. Following incubation of the solidified medium, the number of colonies (CFUs) usually originating from a single cell can be determined.

Surface plating method: As an alternative to the plate-pouring method, a small volume of a microbial suspension is applied to the surface of solid agar plate and evenly spread out e.g. using a so-called Drigalski spatula (see Fig. 2.3), the tip of sterile pipette or sterile glass beads.

Figure 2.3: Drigalski spatula

Membrane filter technique: For this technique already poured plates are used. A known amount of the sample (e.g. 100 ml) is filtered through a membrane filter with a pore size of 0.45 µm on which the microorganisms are retained. The filter is then placed on an agar plate. The medium is passively soaked into the filter. The microorganisms grow on the filter and form colonies which can be counted as in the case of the plate pouring method. With this technique larger microbial samples with a low titer can be examined. This method is used to determine e.g. the *E. coli* content in drinking water.

Many new procedures are being developed in order to check for the presence of specific micro-organisms in water. There are for example **capsules for bacteriological water analysis** whose content is mixed together with the sample in a sterile recipient which is then incubated. This allows for example a qualitative estimate of the presence of *E. coli* based on a colour change similar to the bacterial diagnostic kits used in Experiment 3.5. In this course, we use **TBX agar** plates to assess the presence of *E. coli* in different water samples (Experiment 2.4 and 2.5). TBX agar contains bile salts favouring the growth of Gram-negative bacteria and a chromogenic substrate (5-bromo-4-chloro-3-indolyl-β-D-glucuronide = X-glucuronide) for the detection of the enzyme β-glucuronidase. This enzyme differentiates *E. coli* from most other coliform (lactose-fermenting, gram-negative, rod-shaped) bacteria. Conversion of X-glucuronide by the enzyme leads to a dark blue colour of the *E. coli* colony.

Experiment 2.4: Determination of *E. coli* content in different water samples

Each assistant group determines the total number of aerobic, mesophilic bacteria in three different water samples selected from tap water, mineral water, lake water, river water and

own water samples. Based on the obtained number of *E. coli*-colonies, the drinkability of these waters shall be judged.

Procedure:
100 ml of the water samples are checked for the presence of *E. coli* using the indicator agar TBX and the membrane filter technique (see above).

Results:

Water sample	Number of (blue) *E. coli*-colonies per 100 ml	Safe to drink? (Y/N)*

*Compare the determined germ numbers with the allowed threshold values (see above) for evaluation.

Experiment 2.5: Determination of *E. coli* content in raw water from a sewage treatment plant

Sewage water contains mainly human enteric bacteria including a high concentration of *E. coli*. In this experiment, each student group determines, as a comparison to the water samples analysed in Experiment 2.4, the abundance of *E. coli* in raw water from a sewage treatment plant (ARA Killwangen, Aargau) using again the *E. coli* indicator agar TBX. Since the expected abundance is much higher than in the other water samples, the surface plating technique is applied.

Procedure:
A dilution series (10^{-2}, 10^{-3} and 10^{-4}) of raw water is prepared in sterile water. 250 µl of each dilution are plated on TBX agar plates using the Drigalski spatula. The plates are incubated at room temperature.

Evaluation:
1) From the number of colonies on the TBX plates, calculate the number of *E. coli* (blue) colonies per 100 ml of sewage water: _____ CFU/100 ml
2) Compare the number with the number of non-*E.coli* (non-coloured) bacterial colonies on the TBX plates: What is the percentage of *E. coli* among the enteric bacteria of the sewage water grown under these conditions? _____ %

3) Compare the number with those obtained in Experiment 2.4: How many orders of magnitude does the number of *E. coli* cells of sewage water exceed the worst environmental water sample tested? _____fold

Experiment 2.6: Testing the effect of a filter with pore size 0.45 µm on raw water from a sewage treatment plant

Since most bacteria are larger than 0.45 µm, they can be removed from a liquid using a filter with this or an even smaller (0.2 µm) pore size. The retention of all microorganisms in a filter with pore size 0.45 µm will be tested in this experiment on the supplied raw water from sewage plant.

Procedure:

5 ml of undiluted raw water are filtered through a filter with pore size 0.45 µm into a sterile tube. Afterwards 1 ml of raw water and the undiluted filtrate are checked for the presence of aerobic, mesophilic bacteria by plating on LBG agar using the plate pouring method. Plates are incubated at room temperature. **Do not discard the filtrate – it is used in Experiment 2.7!**

Results:

Sample	**CFU/ml**
Unfiltered raw water	
0.45 µm-filtered raw water	

Experiment 2.7: Determination of the titer of *E. coli*-phages in the 0.45 µm filtrate

Twort (1915) and d`Hérelle (1917) discovered an infectious agent, which could pass bacterial filters and lyse a bacteria colony. They called this agent bacteriophage. Today, we know that these filterable and in the light microscope non-visible bacteriophages are viruses, which can only infect bacteria.

Like other viruses, bacteriophages (or shorter, phages) consist of proteins and nucleic acids. The phages contain either DNA or RNA, never both. During the infection the nucleic acid is injected into the bacterium and provides the information for the synthesis of new phage particles. The energy, the components (nucleotides, amino acids) and the „production sites“ (ribosomes, membrane structures) are provided by the host organism.

Today, bacteriophages have been isolated and described for almost every bacterial species. The host range of a bacteriophage is very restricted; i.e. a given phage species can infect only one or few bacterial species.

The identification and isolation of phages is only easy if the phage is virulent, i.e. if it proliferates in the bacterium just after the infection and finally lyses its host upon release of genetically identical phages. However, there are also temperent phages which inject their nucleic acids into the host bacterium but do not proliferate immediately. In these cases, the nucleic acid of the phage is integrated in the host chromosome and is replicated together with the host chromosome during cell division. Only under special conditions (such as induction with UV-rays or chemical agents) the phage-DNA becomes independent and starts to proliferate independently of the host chromosome: the temperent phage becomes lytic.

In order to determine the number of phages (the phage titer), the plaque-test-method is used. For this method, few phages are mixed with sensitive bacteria in excess, mixed with soft agar and then dispersed on the surface of an agar plate. Wherever a phage particle is located, the bacteria in a certain perimeter are lysed. This way, “holes” are formed in the bacterial lawn – the technical term is plaques. For statistical reasons we assume that every plaque is formed by the infection of a single phage i.e. we can obtain a measure of the number of infectious particles or PFU (plaque-forming unit) in a lysate (phage titer).

For this assay the soft-agar layer method (a modified plate pouring method) is used. The phages are embedded in a soft agar which is at the same time inoculated with the host bacteria (here: *E. coli*). The soft agar promotes the diffusion of the phages in order to contact host cells more easily and to infect the bacteria. After about 20 minutes, 20 to 200 phages have been synthesized in each infected cell. The infected bacteria lyse and release the phages which attack new hosts. Thus, a bacteria-free zone, referred to as halo or plaque, is formed in the dense bacterial lawn during incubation.

Material:

- 0.45µm-filtrate of Experiment 2.6
- Liquid culture of *E. coli* BL21
- 2 tubes with 7 ml of melted H-softagar (in a test tube) kept liquid in a 60°C water bath
- Two prewarmed H-agarplates

Procedure:

- Dilute 50 µl of the 0.45µm-filtrate in 450 µl of sterile water (1:10 dilution) in an Eppendorf tube
- Add the entire volume (0.5 ml) of the diluted filtrate together with 50 µl of the *E. coli* BL21 culture to one of the softagar tubes and mix
- Pour the mixture immediately (before it solidifies) onto a H-agar plate and evenly spread by circular agitation of the plate
- Repeat the procedure with 0.5 ml of the undiluted filtrate
- Incubate the plates at room temperature

Evaluation:

1) Calculated the titer of *E. coli* phages based on the number of plaques (consider the dilution): _____ PFU/100 ml

2) Compare the determined number of *E. coli* phages with the number of *E. coli* cells in the same volume of sewage water determined in Experiment 2.5: Does the order of phages exceed the number of host cells? How do you explain the result?

ANSWER:

Personal notes:

3. MORPHOLOGY AND DIAGNOSTICS OF BACTERIA

Introduction

In order to be able to evaluate the results of a sampling of microorganisms, either from the environment or from a patient suffering from an infection, the identification of the microorganisms is crucial. In this chapter, you will learn some features of bacteria which can be used to distinguish them from each other and to eventually identify them at the species level. You will be able to apply some of the methods to the bacteria that you isolated yourself from the environment.

Morphological criteria

Cell morphology, size and patterns of cell division

Bacterial cells can merely be seen using a light microscope. The diameter varies from species to species and averages at 1 - 2 µm. For the examination of fine structures, an electron microscope is needed. Eukaryotic fungal cells are 5 to 10 times larger (5-8 x 5-15 µm). Bacterial cells divide by fission. Under ideal growth conditions, the fission may occur with a delay and thus characteristic cell aggregates (chains, grapes, cubic packages, plates, tetrads) are formed. Both in bacteria and fungi, multicellular forms exist that form filaments and propagate by spores. Cell division of filamentous fungi is restricted to the tips of the filaments (polar growth, see Chapter 4).

Cell wall

Microbial cells are surrounded by a cell wall which protects the vulnerable protoplast from mechanical damage and bursting under hypoosmotic conditions. The cell wall of bacteria consists of murein, a network of heteropolysaccharide strands and peptide bonds. The cell wall of archaea is built from protein and/or polysaccharides and the one of fungi from polysaccaccharides only. On the basis of the cell wall structure, bacteria can be divided in two important groups, the Gram-positive and the Gram-negative bacteria. Gram-positive bacteria have a thick cell wall, while the cell wall of Gram-negative organisms is relatively thin but has an additional outer membrane consisting of lipopolysaccharides and lipoproteins. The differentiation between Gram-positive and Gram-negative bacteria is made by using a staining technique, the so called Gram stain (see Experiment 3.3). Gram-positive organisms remain coloured after staining with crystal violet and fixing with potassium iodide solution as opposed to Gram-negative bacteria, where the stain can be removed with ethanol.

Capsule or mucus layer

Additionally, cell walls can be surrounded by a capsule or a mucus layer. These are polysaccharides excretions which have an additional protective function for the cell. When the polysaccharides are relatively compact and stick to the bacteria, we refer to them as capsules. In contrast, the watery mucus can be removed relatively easy.

Cytoplasmic membrane

The cytoplasmic membrane is located between the cell wall and the cytoplasm. It consists of a double layer of phospholipids, in which proteins are incorporated. In the first place, the cytoplasmic membrane fulfills a transport function for nutrients and metabolites. In addition, there are also enzymes incorporated which are important for the cell's metabolism.

Cytoplasm

The cytoplasm contains the chromosome, ribosomes, proteins, reserve nutrients and all the other compounds which are important for the progeny of the cell and the formation of cell matter. The cytoplasm of eukaryotic fungal cells contains a number of membrane-surrounded compartments (mitochondria, vacuoles, cell nucleus). Such compartments are not found in bacteria or archaea.

Motility

Many bacteria can move actively. They are equipped with flagella. The anchors of the flagella reach through the cell wall into the cytoplasmic membrane. The composition and function of the flagella is complicated. The arrangement of the flagella on the surface is characteristic and important for taxonomy. There are e.g. bacteria which have only one flagellum on one end (monopolar monotrichous) but there are also the other extreme, bacteria with many flagella all around the cell (peritrichous). Cells of archaea and fungi are usually not motile.

Spore formation

There are two families, the Bacillaceae and the Clostridiaceae, which form heat-resistant endospores at the end of the logarithmic growth phase. The vegetative cells of both families are Gram-positive. The Bacillaceae are aerobic while the Clostridiaceae are strictly anaerobic. Spore formers are very common in nature and include e.g. also the filamentous Actinomycetes and all filamentous fungi. Spore formation starts as soon as the vegetative reproduction stops due to lack of nutrients. The spores can retain viability for years. They germinate if the conditions for vegetative growth become favourable.

The microscope and its use in microbiology

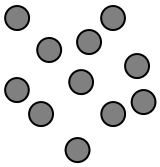
Using the naked eye, microorganisms can only be seen when they are present in large numbers. Clear nutrient media do not show turbidity unless the cell number exceeds 10^6 cells/ml. A colony on an agar plate which is barely visible, consists of approximately 10^5 to 10^6 cells; i.e. a single cell on an agar plate will only be visible as a colony after 15 cell division cycles (see Experiment 4.1). Light microscopy allows the observation of single microbial cells. Different cell shapes can be distinguished and the dimensions of the observed microorganisms can be estimated. Structural details however are hardly visible with light microscopy due insufficient resolution and contrast. The presence of flagella for example can only be deduced because bacteria with flagella show a clearly visible motion. The

microscopes available in the course are equipped with phase contrast which enhances contrast and thus allows the observation of the microorganisms in their native state. Previous methods of contrast enhancement by staining are thus mainly unsuitable.

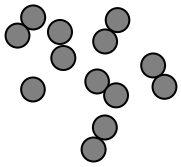
Cell shapes of prokaryotic microorganisms

Cocci:

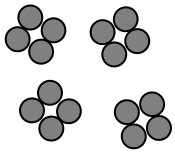
- Spherical cells with a diameter < 2 µm
- Non-motile
- Distinction based on the number of fission layers



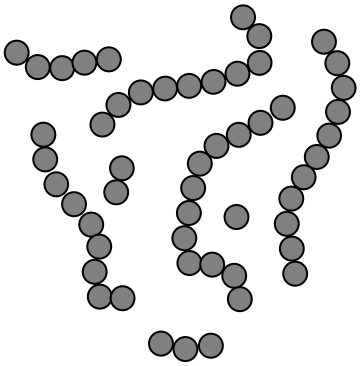
Micrococci: predominantly single cells, rarely larger cell aggregates.



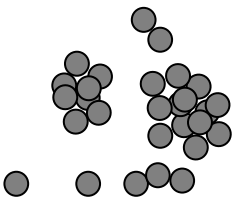
Diplococci: predominantly units of two cells, rarely single cells and larger cell aggregates.



Sarcines: rhombically arranged packages of four cells, rarely other cell aggregates.



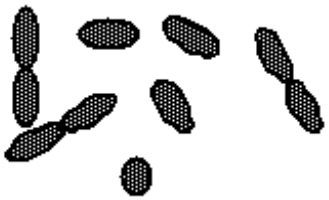
Streptococci: long cell chains accompanied by single cells and shorter cell chains.



Staphylococci: cell clusters accompanied by single cells and small cell clusters.

Rods:

- Large variability of the length/width ratio dimensions: (0.2 - 1) x (1 - 5) µm
- Straight and twisted rods (often not distinguishable in light the microscope)
- Motile or non-motile
- Reproduction by fission

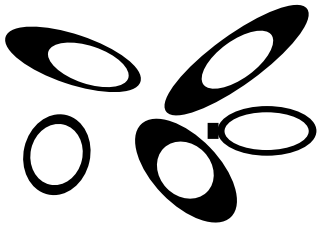


Short rods: single and pairs of dividing cells.

Long rods: Chains of bacteria. The individual cells can be recognized. Chains typical for logarithmic growth phase when the fission drags behind.

Sporulating cells:

Examples: *Bacillus thuringiensis*, *Bacillus anthracis*



- Characteristic refractility (appear bright) of the spores in the phase contrast microscope
- As a rule, cells are elliptical in shape. Characteristic positions of the spores within the sporulated cell (central, sub-terminal, terminal)

Rare bacterial cell shapes

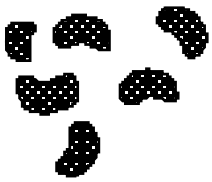
Bacteria show a great diversity of shapes. Rods and cocci are the most common shapes, but there are a number of bacterial genera, whose representatives have very distinctive shapes.

Spirochaeta/Spirosoma

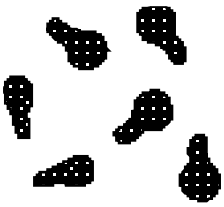
Genera:

Treponema: Causative agent of Syphilis

Borrelia: Causative agent of Lyme disease



Vibrions
Genus: *Vibrio*
e.g. *Vibrio cholerae*: Cholera



Clubbed shaped bacteria
Genus: *Corynebacterium*
e.g. *Corynebacterium diphteriae* (Diphteria-causing agent)

Stalked bacteria
Genus: *Caulobacter*

Filamentous, multicellular bacteria
Genus: *Streptomyces*
NOTE: These bacteria form spores similar to fungi

Guidelines for light microscopy

Main parts of the light microscope

Ocular
Objective
Object table, Cross table
Condensor iris
Coarse and fine focus adjustment wheel
Light field iris

Use of the light microscope

Microscopes are expensive instruments to be handled with care. Please proceed in the following order:

1. Switch on the microscope light.
2. Choose the objective with the lowest magnification which is as a rule 10x.

3. Place the slide with the specimen on the object table. Make sure that there is enough space between the objective and the slide.
4. Move the objective carefully down towards the slide with the coarse focus adjustment until the specimen is in focus. To bring the edge of the coverslip in focus may help.
5. Use the fine adjustment to bring your specimen in optimal focus.
6. If the object is focused you can switch to the higher magnification without changing the adjustments. Some fine adjustments may be needed.
7. Oil immersion is used for microscopy with the 100x objectives. A drop of immersion oil is placed onto the cover slip and the slide is moved carefully back under the 100x objective where the immersion oil fills the space between the cover slide and the tip of the objective.
8. **WATCH OUT THAT OTHER OBJECTIVES (40X; 20X; OR 10X) DO NOT COME IN CONTACT WITH IMMERSION OIL. THE OIL MAY DAMAGE THESE OBJECTIVES IRREVERSIBLY.**
9. The immersion objective has to be cleaned from oil after use with Kleenex paper dampened with ethanol.
10. Clean lenses and oculars eventually using Kleenex paper slightly dampened with water. In particular, immediately remove remnants of culture media from all the lenses.

Examining microorganisms with the microscope

a) Microorganisms grown in liquid culture:
- Dilute culture until a slightly turbid suspension is obtained
- Place a small drop on a microscope slide with a Pasteur pipette
- Apply a cover slip, preferably without trapped air bubbles
- Remove excess water with a Kleenex if necessary (there should be only a thin film of water between the microscope slide and the cover slip, except if you have to check the motility of bacteria
- Examine the specimen

b) Unicellular microorganisms grown on solid media:
- Place a small drop of water on a clean microscope slide
- Remove a bit of cell material from the plate with a toothpick or a loop and mix it with the water drop on the slide until a homogenous slightly turbid suspension is obtained
- Proceed as described above

c) Multicellular microorganisms grown on solid media:
- Transfer a small piece of the mycelium together with the attached agar onto a clean microscopy slide
- Apply a cover slip and spread the filaments by squeezing the agar
- Examine the specimen

Biochemical and genetic criteria

The determination of an unknown microorganism is complex and time-consuming, because one cannot rely exclusively on morphological criteria. Latter criteria are however often sufficient to differentiate between fungi and bacteria. Among bacteria, we can differentiate between cocci, rods and spirilla, and spore formers, or by using selective nutrient media. A very important criterion for bacteria is their Gram-reaction. By means of the Gram-staining, bacteria are divided into Gram-positive and Gram-negative. The next steps in the determination include the testing of several physiological and biochemical properties; e.g. the metabolism of sugars, amino acids as well as other substances which can be used as C- or N-source, gas or acid formation under defined growth conditions, the ability to grow under anaerobic conditions, as well as the resistance to antibacterial substances.

For microorganisms which are important in food microbiology or particularly in medicine, various test systems are offered by several companies. These systems are based on selective media as well as enzymological and sometimes immunological tests. The tests are designed for testing samples with a minimal effort in material and time. However, the tests only yield reliable results when the following conditions are met:

- test organisms must not be contaminated with other microbes (pure cultures)
- test organisms must be fresh
- often separate tests (e.g. Gram-stain, indole test, oxidase test) of test organisms must be performed

In our course, we are working with such a test system, the 'EnteroPluri-Test' distributed by Chemie Brunschwig. The test allows the differentiation between Enterobacteriaceae and other Gram-negative, oxidase-negative bacteria. For the identification of bacteria suited to be analysed by this test, a Gram-stain and an oxidase test are performed. The EnteroPluri-Test itself includes the indole test for the ability to convert tryptophan to indole, a typical feature of Enterobacteriaceae.

Besides these phenotypic tests, genetic tests e.g. PCR-amplification and sequencing of 16S rDNA, have become popular to unequivocally identify a bacterium. The advantage of these tests is that they are not dependent on cultivation (DNA is amplified *in vitro* by PCR) and therefore very fast. The genetic tests rely, however, as the phenotypic tests as well, on reliable databases with data (DNA sequences) of already identified bacteria. We will use this technology in this course to differentiate between aerobic Gram-negative and -positive bacteria.

Diagnostic experiments

8 agar plates labelled with A-H and containing microorganisms to be diagnosed are distributed. The diagnosis is performed in the sequence listed in Table 3.1 (at the end of this

chapter) and the results of the observations and the different tests shall be entered in Table 3.2.

Experiment 3.1: Macroscopic examination

With the naked eye, the colonies on the agar plates are examined. Special attention has to be paid to mycelium formation as well as to colour and morphology of the colonies. These macroscopic features allow first, although rather vage conclusions on the type of microorganism.

Experiment 3.2: Microscopic examination

From each culture, a native specimen for the light microscope is prepared as described above. Cell shape, size and the presence of nuclei or vacuoles are determined.

Experiment 3.3: Gram-stain and growth on MacConkey agar

As previously mentioned, bacteria can be classified in Gram-positive and Gram-negative, based on the structure of their cell wall. In practice, Gram-staining does not always provide unambiguous results, because the transition from red to purple is often not clear-cut. Therefore it is advisable to perform the tests also with reference cultures. The Gram-staining must always be performed with fresh cultures, grown on a suitable medium. Cells are suspended on a microscope slide. Too high concentrations may lead to cell aggregates retaining crystal violet even after intense washing and the may be falsely classified as Gram-positive. On the contrary, damaging of the integrity of the Gram-positive cell wall layer by boiling of the sample may lead to false-negative results. Despite these difficulties, Gram-staining is still an important criterion for differentiation among bacteria in medical diagnostics. Gram-negative bacteria can be isolated selectively by streaking the bacteria on MacConkey agar no. 3. This agar is strictly selective for Gram negative organisms, i.e. all bacteria which grow on this agar are Gram-negative. However this does not mean that all bacteria which do not grow on MacConkey no. 3 are Gram-positive. Some species of the Gram-negative genera *Moraxella*, *Pseudomonas*, *Flavobacterium* and *Pasteurella* do not grow on MacConkey agar.

Solutions and material:

- Gram-staining solutions from Color Gram 2 Kit (Biomérieux)
- 1 MacConkey no. 3 agar plate

Procedure:

1. The cell material from a fresh bacterial culture is applied on the microscope slide with a LITTLE BIT of water by using a loop or a toothpick
2. Air dry the streak out (do NOT HEAT DRY as you may destroy the cell wall structure of Gram-positives and have them appear as Gram-negatives)
3. Overlay one minute with Gentiana-Crystal Violet solution

4. Wash immediately with indirect water jet
5. Overlay one minute with Lugol solution
6. Wash specimen for approx. 5 seconds with a alcohol-acetone (1:1) solution until no blue clouds are released any more
7. Wash immediately with indirect water jet
8. Overlay one minute with Safranin solution
9. Wash immediately with indirect water jet
10. Dry the preparation using filter paper
11. Cover specimen with a cover slip and examine under the light microscope (bright field, 100x objective, immersion oil).
12. Gram-negative bacteria appear red to pink, Gram-positives are blue-violet

Gram-positive and Gram-negative organisms can be stained on the same microscope slide and even in a mixed suspension.

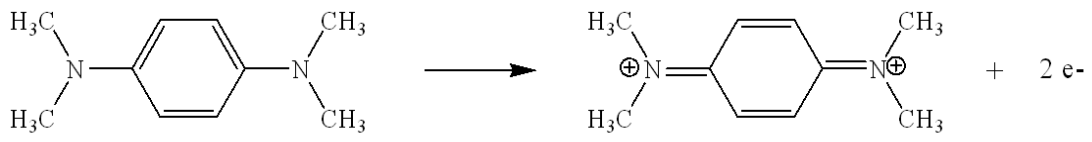
A MacConkey agar plate is divided in sectors. Each sector is inoculated with a little bit of cell material of a bacterial culture. After incubation for 24-48 h at room temperature, the plates can be analysed. MacConkey agar, which contains bile salts and crystal violet, only allows the growth of Gram-negative bacteria.

Experiment 3.4: Oxidase test for the differentiation of aerobic Gram-negative bacteria

The Oxidase test is a biochemical test performed on aerobic Gram-negative bacterial species to determine whether they have cytochromes of the c-type in their respiratory chain. Respiratory chains in the mitochondria of eukaryotes involve cytochromes of the a-, b- and c-type. The respiratory chains of most bacteria contain different enzymes and electron carriers but some bacteria possess cytochromes of the c-type. Pseudomonadaceae are usually Oxidase-positive, whereas Enterobacteriaceae are usually Oxidase-negative.

The reaction:

Step 1



Step 2

Cytochrome c (oxidized) + 2 e^- → Cytochrome c (reduced)

The cytochrome c detection is carried out with N,N,N',N'-tetramethyl-p-phenylendiamine. The redox potential of this easily oxidizable reagent is perfect for donating its electrons to

cytochromes c. The reagent is colourless in its non-oxidized state but rapidly turns blue upon oxidation.

Procedure:

The oxidase test with the Gram-negative bacteria (except the spiral-shaped one) is performed as follows: A filter paper is slightly moistened. An inoculating loop full of bacteria is then applied to the filter paper. The capsule with the reagent inside a protective tube is broken. One drop of reagent is applied to the bacteria. The appearance of a blue color within ten seconds indicates a positive reaction. Record the result in Table 3.2.

Experiment 3.5: EnteroPluri-Test for the identification of Gram-negative, oxidase-negative bacteria

As an exemplary biochemical diagnostics tool of this course, the above described EnteroPluri-Test will be used to identify the bacterium previously identified as Gram-negative and oxidase-negative, at genus or species level. Similar kits exist for other types of bacteria and they all include arrays of different biochemical reactions that allow the differentiation between different bacterial species (see e.g. http://www.bd.com/ds/productcenter/is-crystal.asp). The reactions are all based on the microbial utilization and degradation of specific substrates which are detected by various (mostly pH) indicator systems (see table below and http://www.bd.com/ds/productCenter/245000.asp for further details). In contrast to above oxidase test, these tests require longer incubation times and, thus, can only be evaluated in the next course.

Procedure:

1. Remove both caps of the system. Using the tip of the inoculation needle, placed under the white cap, pick up a well isolated colony from the agar surface without penetrating into the agar.
2. Inoculate EnteroPluri-Test by turning and withdrawing the needle throughout the sectors of the system.
3. Reinsert the needle with a turning movement until the breakage notch; break the inoculating needle folding it in correspondence with the notch. The portion of the needle remaining inside the system keeps anaerobic conditions necessary for reactions of the sectors **Glucose/Gas**, **Lysine** and **Ornithine**.
4. Use the broken portion of the needle, remained in the user hands, to punch the plastic film in correspondence of the holes of the sectors **Adonitol**, **Lactose**, **Arabinose**, **Sorbitol**, **VP**, **Dulcitol/PA**, **Urea**, **Citrate** in order to support aerobic growth.
5. Screw on again both caps and incubate the tube at 37°C for 18-24h putting it on its flat surface.

Evaluation:

1. Observe the change in color of culture media in the different sectors (see table below) and interpret the results using the distributed Evaluation sheet. *NOTE: The VP test is not used in our case.*

Sector	BIOCHEMICAL REACTIONS	Sector colour	
		Positive reaction	Negative reaction
Glucose / Gas	Glucose fermentation	yellow	red
	Gas production	lifted wax	overlaying wax
Lysine	Lysine decarboxylation	violet	yellow
Ornithine	Ornithine decarboxylation	violet	yellow
H_2S / Indole	Hydrogen sulphide production	black-brown	beige
	Indole test	pink-red	colourless
Adonitol	Adonitol fermentation	yellow	red
Lactose	Lactose fermentation	yellow	red
Arabinose	Arabinose fermentation	yellow	red
Sorbitol	Sorbitol fermentation	yellow	red
VP	Acetoin production	red	colourless
Dulcitol/PA	Dulcitol fermentation	yellow	green
	Phenylalanine deamination	dark brown	green
Urea	Urea hydrolysis	purple	beige
Citrate	Citrate utilisation	blue	green

2. Record the obtained results on the evaluation sheet except the Indole test. This test requires injection of a special reagent, the Kovacs reagent, into the corresponding chamber. The test is positive if the injected reagent turns from colourless to pink-red in 10-15 sec.

3. Form the 5-digit code by summing up the numbers in the five groups on the evaluation sheet.

4. Identify the bacterium using the Codebook.

Experiment 3.6: Amplification and sequencing of 16S rDNA for the identification of Gram-positive and -negative bacteria

16S ribosomal RNA (or 16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes. The genes coding for this RNA are referred to as 16S rDNA and are used in reconstructing phylogenies, due to their high degree of sequence conservation between different species of bacteria and archaea. In addition to highly conserved flanking regions, the gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rDNA sequencing has become prevalent in medical microbiology as a rapid and cheap alternative to phenotypic methods of bacterial identification. Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species or even genera. It has also been used to describe new species that have never been successfully cultured. Type strains of 16S rRNA gene sequences for most bacteria and archaea are available on public databases such as NCBI (http://www.ncbi.nlm.nih.gov/). However, the quality of the sequences found on these databases is often not validated. Therefore, secondary databases which collect only 16S rDNA sequences are widely used. The most frequently used databases are Ez-Taxon-e (http://www.ezbiocloud.net/ - *requires*

member login), Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp), SILVA (http://www.arb-silva.de) and GreenGenes (http://greengenes.lbl.gov/cgi-bin/nph-blast_interface.cgi). Latter website (TYPE THE ENTIRE ADDRESS IN YOUR BROWSER) is recommended for this course.

Per student group, two PCR-reactions are set up. For analysis, any of the bacteria isolated during the experiments of Chapt. 2 or any of the bacteria distributed in the course of this chapter and not analyzed by the EnteroPluri-Test can be chosen. The financial resources of the practical course allow the sequencing of one PCR product per student group.

NOTE:
- The sequencing of your samples is sponsored by Microsynth AG (Balgach, Switzerland).
- The primers used work for many but not all bacteria.
- The accuracy of the identification depends on the quality of the databases used.

Material (on ice):
- PCR-reaction mix consisting of (per 50 µl):
39.5 µl sterile distilled water
5 µl 10X *Taq* DNA polymerase buffer
1 µl 10 mM dNTPs (10 mM each dATP, dTTP, dGTP. dCTP)
2 µl 10 µM forward primer (27f, AGAGTTTGATCMTGGCTCAG)
2 µl 10 µM reverse primer (1492r, CGGTTACCTTGTTACGACTT)
- *Taq* DNA polymerase (purified during P GL Bio I, Biochemistry part)

Procedure (all steps but last on ice):
- Label a 0.5 µl PCR-tube with a prenumbered sticker.
- Pipet 50 µl of PCR-reaction mix into the bottom of the tube.
- Add a SMALL amount of a fresh colony to the reaction mix in the tube: GENTLY (you are more likely to pick up too much bacteria than too little) touch the colony with a yellow pipette tip attached to the P200 pipette and release the bacteria by dipping the tip into the reaction mix. You are free in your choice of bacterium to be identified but please fill the information about the origin of the analyzed bacterium into the sample info sheet as a basis of our decision which PCR fragment will be sent for sequencing (in case both bacteria yield a fragment).
- Add 1 µl of Taq DNA polymerase (avoid any drops of enzyme at the outside of the pipet tip) directly to the reaction mixture, mix by flicking the tube with your finger and place the tube in the precooled block of the PCR machine
- As soon as all the tubes of the different groups are ready, start the PCR machine and follow the progression of the reaction on the screen. The cycle of the machine is set as follows:

5 min at 95°C
20 sec at 95°C
20 sec at 54°C } 35 cycles
2 min at 72°C
5 min at 72°C

Evaluation:

5 µl of our reactions will be run out on a 1% Agarose gel to check whether the reactions yielded a product (1450 bp DNA fragment). One positive reaction of each student group will be precipitated and sent for sequencing using 1492r as sequencing primer. A picture of the agarose gel and the sequencing results will be sent to you by e-mail. The bacteria can be identified by entering the sequences into one of above mentioned databases (recommendation: GreenGenes).

Document the results for your self-isolated bacteria from the environmental plates (Experiments of Chapt. 2) in the table below and for the bacteria among provided microorganisms in **Table 3.2** (at the end of this chapter).

Source	Isolation procedure	Macroscopic and microscopic characterization	Identity according to 16S rDNA sequence

IMPORTANT: A separate report of this experiment has to be prepared as part of the 'Leistungskontrolle' of this practical course. Take a PICTURE OF THE ANALYZED BACTERIAL COLONY using your smartphone and include it in the report.

Table 3.1:

PROCEDURE FOR THE DETERMINATION OF AN UNKNOWN, AEROBIC MICROORGANISM

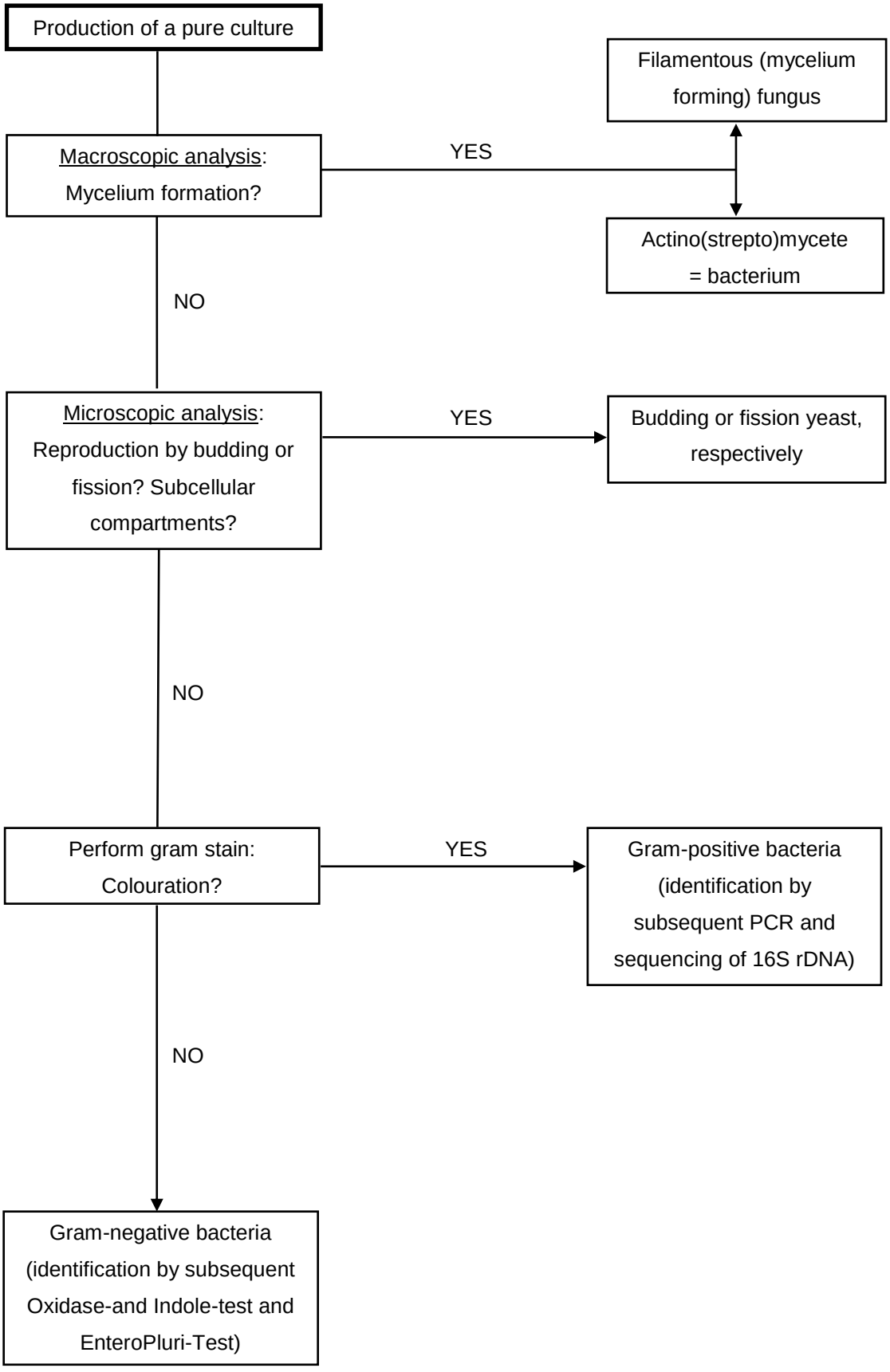


Table 3.2: Evaluation of the results of the diagnostic experiments

Strain	Macroscopic morphology: Growth, colony shape	Microscopic morphology: Cell shape, size, motility	Gram stain	Growth on MacConkey agar	Identified as
A					
B					
C					
D					
E					
F					

4. MORPHOLOGY OF FUNGI

Introduction

Fungi are eukaryotic microorganisms forming their own kingdom besides plants, animals, bacteria and archaea („The fifth kingdom“). Fungi are heterotrophic organisms and play a vital ecological role as decomposers of dead organic material (saprophytes) as well as symbionts (mycorrhiza) and parasites (mycoses) of plants (and animals). They feed by absorption i.e. they secrete proteins (enzymes) degrading the food and absorb the degradation products. Other important characteristic features of fungi are the chitin-containing cell wall and the distribution via spores. Finally, fungi are, as eukaryotes, able to reproduce sexually as well as asexually. Fungi are therefore characterised by an extreme and often confusing diversity of life-cycles and -forms. Fungi are classified according to the type of sexual spore formation (see below).

Fungi play an important role in food microbiology for pre-processing (easier digestibility) and conservation of food (beer, wine, bread, cheese, soybean products). On the other hand large amounts of food (cereals, fruits) are destroyed by fungi each year. Furthermore there are fungi which produce dangerous toxins (e.g. aflatoxin) which have a serious impact on human and animal health.

The aim of this course is to get to know the main fungal phyla and their characteristic morphological features and to familiarise with the fungal lifestyle (see also Chapt. 6).

Vegetative growth

Fungi grow either multi-cellularly as hyphae or by budding or fission of single cells.

Filamentous (multicellular) fungi

Hyphae are filaments with a thickness of 1 - 20 µm, which are formed by linearly arranged cells and surrounded by a cell wall. For the most part, the cells are separated by crosswalls (septum, pl. septa) but are interconnected via septal pores to ensure distribution of nutrients and organelles within the hyphae. A hyphal cell can contain one or more cell nuclei, which can only be made visible using special colouring techniques (Fig. 4.1A). Hyphae branch and form a network which is called mycelium and is usually visible with the naked eye (Fig. 4.1B). Mycelium growth only takes place at the tips of the hyphae (apical growth). The whole fungal body, consisting of the vegetative mycelium with the differentiated reproduction organs, is called thallus.

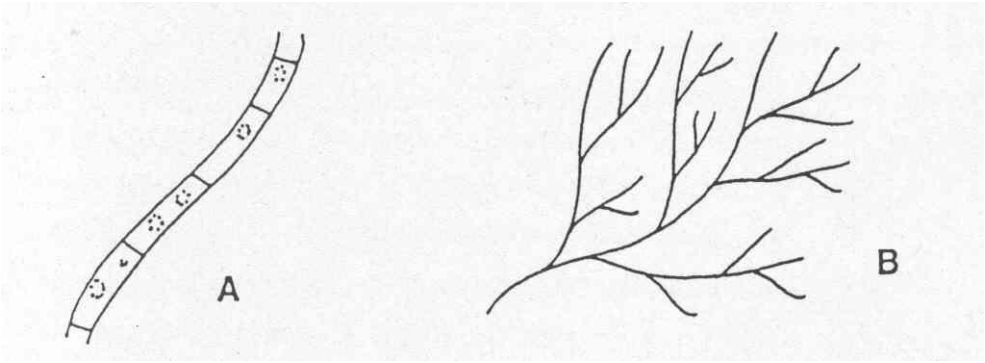


Figure 4.1: Filamentous fungal growth (multi-cellular). A: Piece of a hypha with septa and cell nuclei (dotted); B: Piece of mycelium, consisting of branched hyphae.

Yeast-like (unicellular) fungi

Unicellular fungi proliferate by budding or fission. Budding yeasts (e.g. *Saccharomyces cerevisiae*) develop daughter cells from the mother cell. The two are then separated, leaving scars (Fig. 4.2a, 4.2b). In the case of fission yeasts (e.g. *Schizosaccharomyces pombe*) cell division proceeds similar to bacteria (Fig. 4.2c). Some filamentous fungi (e.g. *Rhizopus* sp., *Mucor* sp.) grow as unicellular organisms (yeast-like) in special development stages or under special ecological conditions (yeast-mycelium-dimorphism).

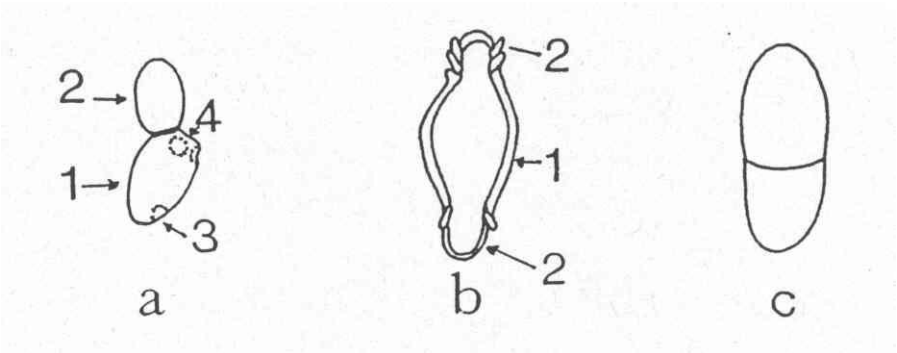


Figure 4.2. Yeast-like growth (unicellular). a: multipolar budding (*Saccharomyces*); b: bipolar budding (*Saccharomycodes*); c: cell fission (*Schizosaccharomyces*). 1: Mother cell, 2: Daughter cell, 3,4: Bud scars.

Spore formation

Fungi ensure their long-term survival and their spatial distribution by the use of spores. The complicated differentiation process either leads to asexual or sexual spores. The formation of asexual spores does not involve a change in the karyophase in contrast to the sexual spore formation. Sexual spore formation involves fertilisation and meiosis.

Depending on the fungi species only the asexual or the sexual or both spore-forming forms are known. That is why a fungus is often known under two names: one name is used for the asexual form (Anamorph), the other for the sexual form (Teleomorph). Both forms characterize a fungal species (Holomorph).

Example: *Fusarium graminearum* and *Gibberella zeae* refer to the same fungus. The name *Fusarium* is used to designate the asexual sporulation form, while *Gibberella* designates the sexual form. In this case *Fusarium* is called the anamorphic form of *Gibberella*.

Forms of asexual spore formation

Endogenous spore formation: This type is characterized by sporangia inside of which the sporangiospores are formed (e.g. in *Rhizomucor miehei*, *Rhizopus* sp. = Zygomycota). Sporangia emerge at the top of hyphae that differentiated to sporangium carriers (sporangiophores). Often a bubble-shaped protuberance (Kolumella) is looming into the sporangium (Fig. 4.3A).

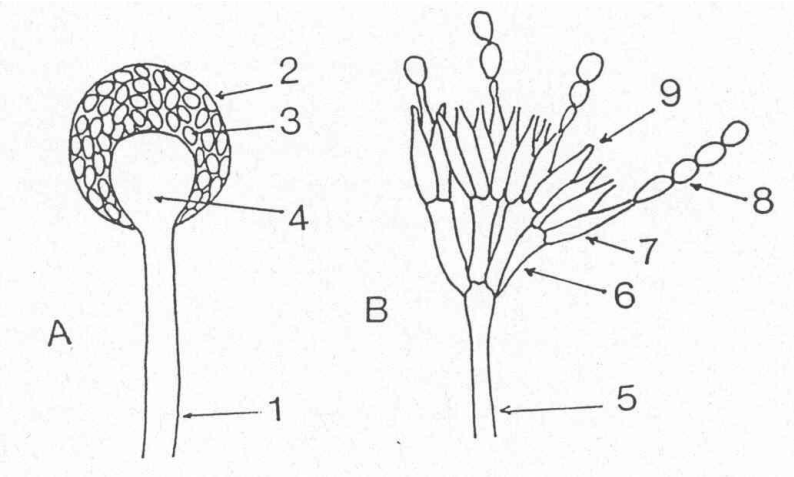


Figure 4.3: Forms of asexual spore formation. A. Sporangium with endogenous spore formation (*Rhizopus nigricans*). 1: Sporangium carrier; 2: Sporangium with sporangiospores (3); 4: Kolumella. B. Conidia carrier with exogenous spore development (*Penicillium* sp.). 5: Conidia carrier; 6: Metulae (1st grade branching); 7: conidiogenic cells, called phialids in this case , 2nd grade branching); 8: Conidiae (in chains) which are formed from the phialid apex.

Exogenous spore formation: In this situation conidiospores are formed exogenously on spore producing cells (phialides; see Fig. 4.3B). The morphogenesis of the conidia differs between different species. In the case of *Penicillium* the conidiophores branch at the top so that the chains of conidiospores form a dense brush (=Pinsel) (Fig. 4.3B).

Forms of sexual spore formation

Basically, three forms of sexual spore formation are differentiated:

Zygospores (*Rhizomucor miehei*). The formation of zygospores occurs in a Zygosporangium (Fig. 4.4C).

Ascospores (*Sordaria macrospora*). The formation of ascospores is carried out in an Ascus (Fig. 4.4A).

Basidiospores (*Coprinopsis cinerea*). The formation of basidiospores is carried out exogenous on a Basidia (Fig. 4.4B).

In the two latter cases, karyogamy does not take place directly after the fertilisation. The two heterosexual cell nuclei remain separated and proliferate via mitosis. This state (karyophase) is called dikaryotic. With Ascomycota, this phase is limited to the ascogenic hyphae inside the fruiting body (Ascom with Asci), whose formation ties up to the plasmogamy. In this state, Basidiomycota can proliferate vegetatively as a dikaryotic mycelium and the formation of a sexual fruiting body (Basidiom with Basidiae) only occurs under certain environmental conditions. The second phase of the fertilisation takes place in the ascus or in the basidia respectively, where fusion of the two nuclei occurs. The diploid nucleus formed divides meiotically afterwards. If a mitosis follows, 8 (Ascomycota), if not 4 (Basidiomycota) haploid nuclei result.

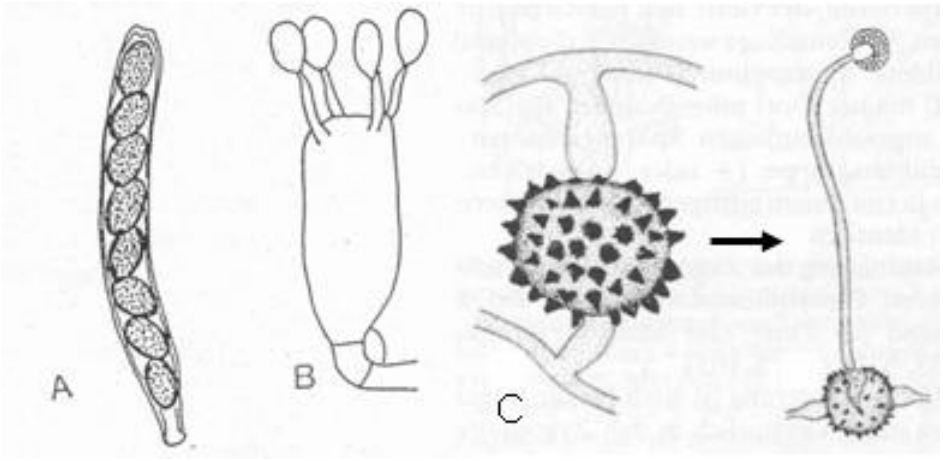


Figure 4.4: Forms of sexual spore formation. A. Ascus with 8 endogenous ascospores (*Sordaria macrospora*); B. Basidia with four exogenous basidiospores developing on sterigms (*Coprinopsis cinerea*). C. Zygospore with endogenous formation of a meiospore, which then germinates into a sporangium (zygosporangium) (*Rhizomucor miehei*).

Experiment 4.1: Determination of the cell number of a yeast colony

Aim:

In order to get a better idea of the size of microorganisms and as an introduction into the microscopy of microorganisms, the cell number of a yeast colony shall be determined using a so-called Neubauer-Counting chamber and dilution plating.

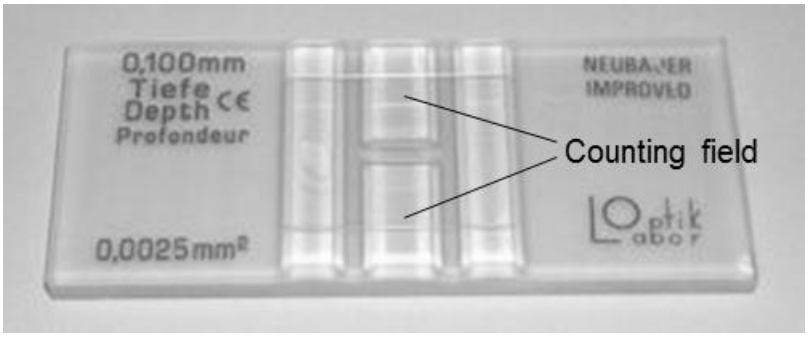
Material:

Per group of 2 students:

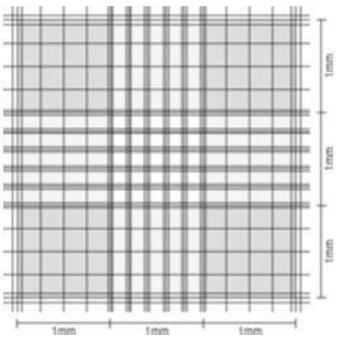
- Yeast (*Saccharomyces cerevisiae*) colony on YPD plate (complete medium)
- Neubauer(improved)-Counting chamber with special cover slide (PLEASE WASH CHAMBER AND COVER SLIDE AFTER USE AND PUT THEM BACK IN PLASTIC BOX!)
- 3 empty YPD plates
- Petri dishes with ethanol and Drigalski spatula for plating

Procedure Counting chamber:

1. The cells of a single yeast colony are resuspended in a sterile Eppendorf cup in 500 µl of sterile water.
2. A cover slip is put on the chamber where the outer footbridges have been wettened. Interference lines (Newton rings) should be visible.
3. App. 10 µl of the cell suspension (or of a 1:10 dilution thereof if the cell number is too high to be counted) is pipetted in between cover slip and chamber by capillary force.
4. The cells of 5 group squares inside of the central counting square (see Fig. 4.5) are counted (cells on the upper and left border are counted as well) and the average cell number is calculated. This number multiplied with 250 corresponds to the cell number per µl of the counted cell suspension. Calculate the number of cells for the whole colony.



Counting field with central counting square



Counting square with 25 group squares

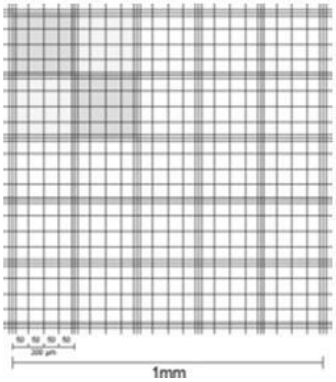


Figure 4.5: Neubauer (improved) counting chamber with counting grid. Note the scale – the side length of the smallest square is 40 µm!

Procedure dilution plating:

1. Prepare a 1:100, a 1:1000 and a 1:10000 dilution of above cell suspension in 500 µl sterile water and plate 250 µl of each on a YPD plate.
2. Incubate the plates at 30°C over the weekend.
3. The calculated total number of colonies corresponds to the number of (viable) cells in the colony.

Results:

Counting chamber: _____ yeast cells per colony

Plating: _____ yeast cells per colony

Experiment 4.2: Microscopy of characteristic morphological features of different fungal phyla

Aim: Microscopic examination of the structures displayed in Figures 4.1 to 4.4.

Fungi to be microscoped (Kingdom: EUMYCOTA) and their features:

Division: ASCOMYCOTA (Sac fungi)

1) *Saccharomyces cerevisiae* (Sc) (Bakers-, Brewers- or Wine-yeast)

Material: 2 different Agarplates (YPD and HSpo) with dry yeast from Migros

Preparation: Cell suspension in water under microscope with phasecontrast and 400x magnification

Features: asexual propagation by budding (YPD), sexual propagation by 4 ascospores in „naked“ ascus (Spo)

2) *Sordaria macrospora* (Sm)

Material: Agarplate with mycelium and black, small pearshaped fruiting bodies

Preparation: Harvesting of fruiting bodies under binocular, squeezed fruiting bodies in wate runder microscope with phasecontrast and 100x to 400x magnification

Features: no propagation by asexual spores known, sexual propagation by 8 ascospores in in fruiting body (perithezium) embedded ascus

3) *Aspergillus oryzae* (Ao) (Green mold)

Material: Agarplate and agar slide

Preparation: Agarplate under binocular, agar slide under microscope with phasecontrast and 400x magnification

Features: septate mycelium, asexual propagation by conidiospores

Division: ZYGOMYCOTA

4) *Mucor rouxii* (Mr)

Material: Agarplate and agar slide

Preparation: Agarplate under binocular, agar slide under microscope with phasecontrast and 400x magnification

Features: non-septate mycelium, asexual propagation by sporangiospores

5) *Phycomyces blakesleeanus* (Pb)

Material: Agarplate with 2 mating partners (AxB)

Preparation: Agarplate under binocular

Features: asexual propagation by sporangiospores, sexual propagation by zygospores

Division: BASIDIOMYCOTA

6) *Agaricus bisporus* (button mushroom)

Material: Agarplate, fresh mature fruiting bodies

Preparation: Sporeprint and microscopy (phasecontrast and 400x magnification) of basidiospores by resuspension of part of a lamella in sterile water.

Features: no known propagation by asexual spores, sexual propagation by 2-spore basidia on lamella at the lower side of the mushroom

5. ANTIMICROBIAL AGENTS

Antibiotics and chemotherapeutics

Among the many microorganisms there is a relatively small number of organisms that are pathogenic to humans, animals or plants. They are a very important topic in human and veterinary medicine and in plant pathology. Antibiotics and chemotherapeutics are antimicrobial substances that can be used to fight infectious diseases. Antibiotics are produced by microorganisms and the basis of this strategy. Chemotherapeutics are synthetic compounds with antimicrobial properties. The transition to the antibiotics however is smooth, because a lot of antibiotics are chemically modified in order to increase their effect or to stabilise them.

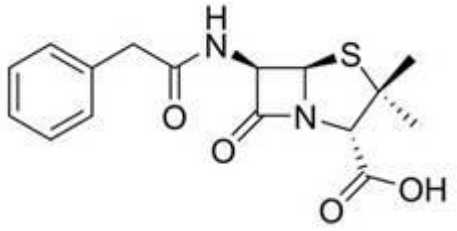
Most microorganisms that secrete antibiotics as secondary metabolites belong to the group of *Aspergillales* (filamentous fungi), to the actinomycetes (filamentous bacteria) or to a few other groups of bacteria. Of the thousands of antimicrobial substances that have been identified to date, comparably few are suitable for therapeutic use. These substances have revolutionized modern medicine as they allow to fight infections caused by bacteria and fungi. Dosage and duration of the therapy must be chosen in a way that all infectious agents are killed. If only a partial elimination of the agent occurs, there is the risk of resistance generation. At the same time, possible side effects towards the host should be minimized.

Microorganisms which form antibiotics have an important advantage in their habitat in that they are able to repress or block the development of other organisms. This results in an increased availability of nutrients. In order for this strategy to work, the producer of the antibiotic must be resistant to its own agents. This is the origin of many resistances since the involved genes are often located on mobile genetic elements (plasmids) and can be transferred to other microorganisms.

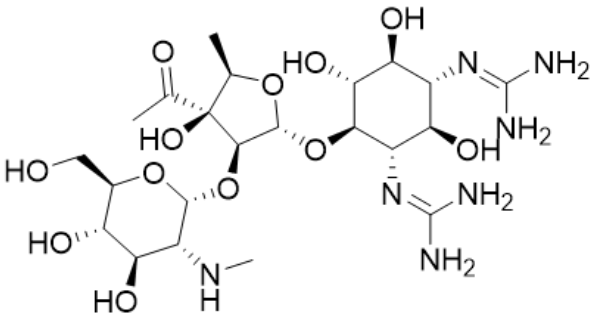
The resistance to antibiotics and chemotherapeutics has become a major problem in medicine. Such resistances can be due to several different mechanisms e.g. the inactivation of the antibiotic by chemical modification of the antibiotic, a change in the antibiotic target, efflux pumps, usage of alternate metabolic pathways, or the bacteria might hide from antibiotics by forming biofilms. The majority of the resistances are plasmid encoded and are thus easily transferred from one microorganism to the other. The plasmid encoded antibiotic resistances are usually based on a chemical modification of the antimicrobial agent. As examples, chloramphenicol is acetylated and penicillin is cleaved by penicillinase. An accumulation of resistant microorganisms can be observed in hospitals. Of particular concern are pathogens that have acquired multiple resistances such as MRSA (Methicillin-resistant *Staphylococcus aureus*) which has become a major threat in many hospitals.

List of antibiotics used in the course

Penicillin G

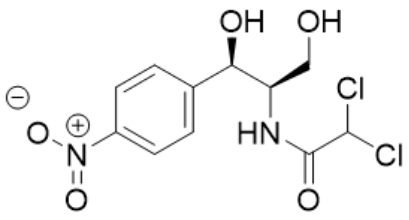


First known antibiotic, discovered 1929 by A. Fleming. Produced by the mold *Penicillium notatum*. Inactivates irreversibly the transpeptidase catalyzing the last step in cell wall biosynthesis of bacteria (crosslinking of the peptide side chains of the murein skeleton).
Medical indications: All sorts of infections
Medical indications: All sorts of bacterial infections



Streptomycin

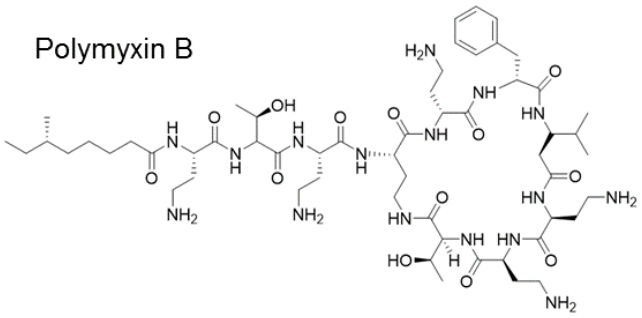
Discovered by S. A. Waksman in the early 40ies. Represents the first effective drug against tuberculosis. Resistances rapidly develop and application can lead to deafness. Produced by *Streptomyces griseus*. Inhibits protein synthesis (initiation, elongation) by binding to protein S12 of the 30S subunit of 70S ribosomes.



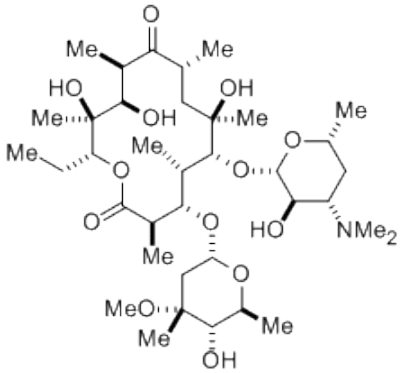
Chloramphenicol

Produced by *Streptomyces venezuelae*, today synthetically produced. Binds to the 50S subunit of the 70S ribosome and inhibits the peptidyltransferase.
Medical indications: Typhus, Salmonella infections etc.

Polymyxin B



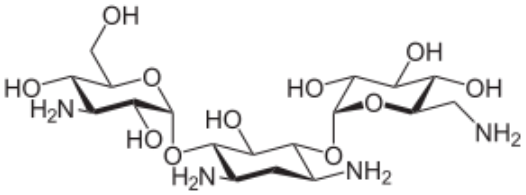
Bacteriocidal peptide-antibiotics mainly used against gram-negative bacterial pathogens. Basic polypeptide acts as a detergent due to an attached fatty acid and permeabilizes the cell membrane. Thick murein layer of gram-positive bacteria is not permeable for PMB. Is synthesized by a non-ribosomal peptide synthetase of *Bacillus polymyxa*.



Erythromycin

Macrolid-antibiotic of similar broad range spectrum against Gram-positive bacteria as Penicillin. Is used in case of allergies against Penicillin. Blocks the exit of the nascent polypeptide chain on the 50S subunit of the bacterial ribosome. Is synthesized by the actinomycete *Saccharopolyspora erythraea*.

Kanamycin and derivatives



Aminoglycoside-antibiotic with broad specificity against bacteria. Is mainly used for local therapy of bacterial eye infections. Binds to the 30S subunit of the bacterial ribosome and inhibits protein biosynthesis. Certain derivatives such as geneticin (G418) also inhibit eukaryotic protein biosynthesis. Kanamycin is produced by the actinomycete *Streptomyces kanamyceticus*.

Experiment 5.1: Fungi as producers of antibiotics

The first industrially produced antibiotic, Penicillin, was discovered 1928 by accident by Alexander Fleming and initiated a new area in fighting bacterial infectious diseases. In 1945,

Fleming received the Nobel Prize in Medicine for his discovery. Penicillin is produced by the fungus *Penicillium chrysogenum* (*notatum*).

In this experiment, the effect of Penicillin-producing *P. chrysogenum* on the growth of *E. coli* is demonstrated.

Material:

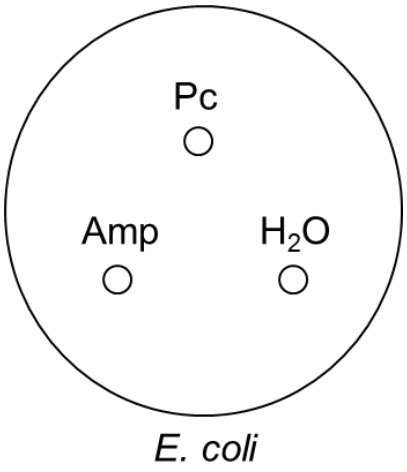
- 1 Agarplate with *Penicillium chrysogenum*
- 2 Test tubes with LBG-soft-agar kept at 60°C
- Eppendorf tubes with ampicillin stock solution (100 mg/l)
- Test tubes with overnight cultures of *E. coli*
- Sterile filter disks

Procedure:

- Mix 100 µl of the bacteria suspension with liquid LBG soft agar.
- Pour the mixture on a plate with *P. chrysogenum* colonies and distribute evenly.
- Using sterilized forceps, place sterile filter disks supplied with 20 µl of H_2O and ampicillin onto the plates according to the scheme below.
- After 2 days of incubation at 30°C, inhibition zones become visible at the locations where antibiotics were secreted.

Evaluation:

Draw borders of the observed inhibition zones in the scheme below.



Experiment 5.2: Susceptibility of different microorganisms towards different antibiotics

The differences in cell structure and composition of Gram-positive and Gram-negative bacteria and fungi lead to different sensitivities of these microorganisms towards antibiotics. This difference shall be demonstrated in this experiment using the two bacteria *B. subtilis*

(Gram-positive) and *E. coli* (Gram-negative) and the yeast *S. cerevisiae* and the three antibiotics Polymyxin B, Erythromycin and Geneticin (G418).

Material:

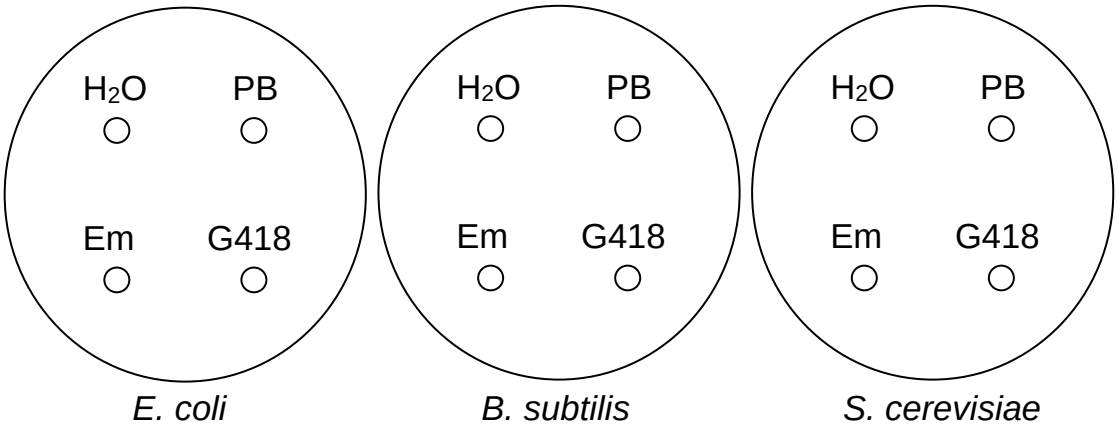
- 2 LBG-agar plates
- 1 YPD-agar plate
- 2 Test tubes containing 5 ml LBG soft agar each
- 1 Test tube containing 5 ml YPD soft agar
- Liquid cultures of *B. subtilis* and *E. coli and* S*. cerevisiae*
- Sterile paper disks
- Sterile water
- Sterile petri dishes
- Stock solutions of Polymyxin B (2 mg/ml in water) and Erythromycin (1 mg/ml in water) and G418 (50mg/ml in H_2O)

Procedure:

Distribute 100 µl of bacterial/ yeast culture each (shake before use) in soft agar on the 3 agar plates. For the bacteria LBG agar is used and for the yeast YPD agar is used. Place 3 paper disks each with 20 µl water, Polymyxin B, Erythromycin and/or G418 in petri dishes. Place the disks on the agar plates according to the scheme below using sterilized forceps. Incubate the plates at 30°C for 2 days.

Evaluation:

Draw borders of observed inhibition zones into the scheme below. Explain the obtained results with regard to the description of the antibiotics in the introduction.



Plant-derived antimicrobial agents

Not only antibiotics and chemotherapeutic agents, but also components of spices can have antimicrobial effects. Tropical plants may protect their seeds with these substances from spoilage with microorgansims. It is not by accident that in the kitchens of tropical countries, where high temperature and humidity favours the growth of microorganisms, the use of hot spices is much more common than in Europe. Spices with proven antimicrobial effect are for instance mustard, garlic, chilli and cloves. It is assumed that secondary metabolites such as phenols, flavonoids and carotinoids are responsible for the antimicrobial effects of the spices.

In the following experiment the antimicrobial effects of mustard and garlic is demonstrated.

Experiment 5.3: Antimicrobial effect of mustard and garlic

Material (per group of 2 students):

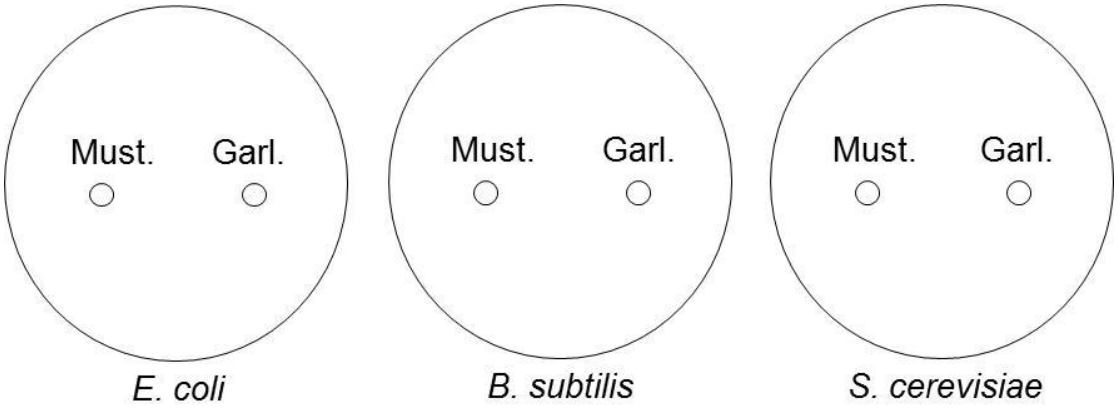
- Petri dish
- 2 LBG agar plates, 1 YPD agar plate
- 2 tubes with LBG soft agar, 1 tube with YPD soft agar
- *E. coli*, *B. subtilis* and *S. cerevisiae* liquid cultures
- Fresh garlic and garlic press
- Mustard (hot)
- Pasteur pipettes

Procedure:

- Liquid LBG- and YPD-soft agar (kept at 60˚C) is thoroughly mixed with 100µl *E. coli*, *B. subtilis* and *S. cerevisiae* liquid culture, respectively. The suspensions are poured onto LBG- and YPD-agar plates, respectively. The plates are put aside to allow the soft agar to solidify.
- The garlic is crushed into the empty petri dish using the garlic press.
- Using the back end of a Pasteur pipette to punch holes in the centre of each agar plate.
- The hole is filled with mustard or crushed garlic (using a flamed forceps).
- The plates are incubated overnight at 30°C.

Evaluation:

A clear zone around the mustard or garlic-containing hole indicates the presence of antimicrobial substances. Indicate the observed inhibition zones in the scheme below:



Animal-derived antimicrobial enzymes

Besides secondary metabolites and peptides, enzymes can have antimicrobial activity and protect from microbial infection. The best known example is lysozyme (Muramidase), a widely occurring enzyme which breaks down the murein in bacterial cell walls. In plants and animals, lysozyme is part of the innate immune system against bacteria. It can be detected in tissues and secretions of the human body (tears, nasal mucosa, saliva, urine, breast milk). Egg white is particularly rich in lysozyme to protect the embryo from bacterial infection. Sequence and three-dimensional structure of the 129 amino acids enzyme are known. Lysozymes are also be produced by phages supporting the release phages from the bacterial host cells or by bacteria during cell division.

After degradation of bacterial cell walls by lysozyme, the naked cells (protoplasts) swell until they burst. Only in iso- and hypertonic media, the spheroidal protoplasts are stable. Cell walls of Gram-negative bacteria consist only to a minor extent of murein. Lipoproteins and lipopolysaccharides form the outer layers. Therefore, lysozyme-mediated lysis of Gram-negative bacteria like *E.coli* is limited.

In the following experiment, the lysozyme activity in different animal and human secretions is detected using a commercially available preparation of dead Gram-positive bacteria.

Experiment 5.4: Detection of lysozyme activity in animal secretions

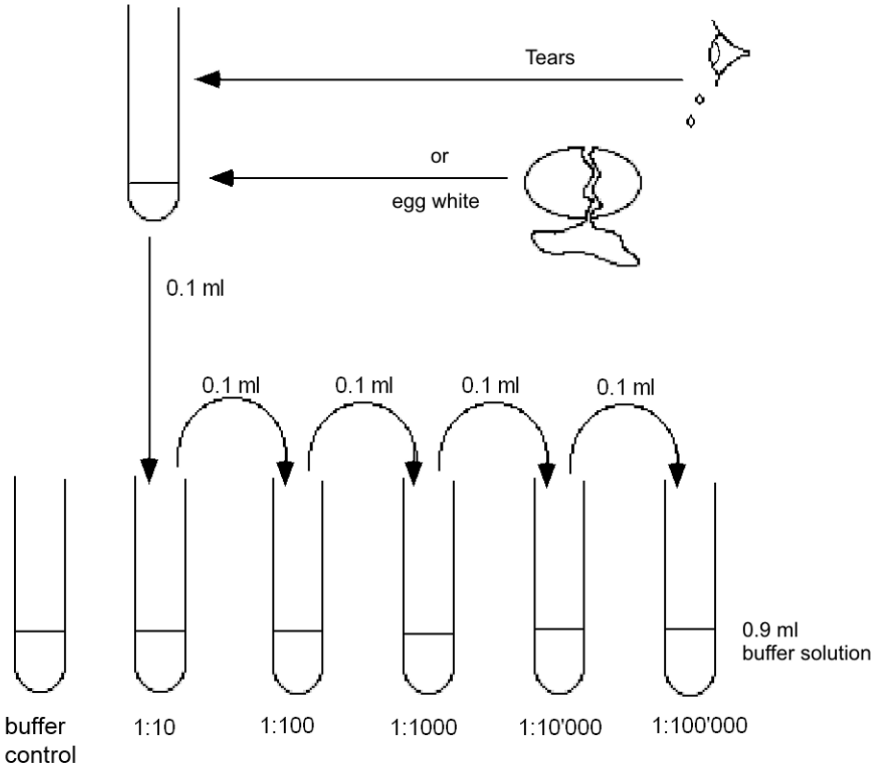
Material:

– Sources of lysozyme: Tears, saliva, egg white
– Five short test tubes with 0.9 ml buffer solution (sodium phosphate buffer 0.1 M, pH 6.0)
– *Micrococcus luteus* (Gram-positive)-suspension as indicator bacterium (2.0 mg/ml)

Procedure:

- Tears, saliva or egg white are diluted according to the scheme below. Tears can be produced by chopping onions and collected using an Eppendorf tube (DO NOT TRY TO PIPET TEARS FROM YOUR EYE - YOU RISK TO SERIOUSLY INJURE YOUR EYE!). Three drops of tears correspond to approx. 0.1 ml. After breaking the egg, place the egg white in a petri dish and take 0.1 ml with a suitable pipette. The opening of pipette tips can be enlarged by cutting it with scissors.
- Add 0.1 ml *Micrococcus luteus* (Gram-positive)-suspension to all 6 tubes
- Determine after 60 minutes at room temperature if the tube is clear i.e. whether lysis has occurred

Scheme of procedure:



Evaluation:

Estimate the relative lysozyme content of the three sources based on the results obtained in the assistant group:

Source	**Lysozyme activity (+++ / ++ / +)**
Tears	
Saliva	
Hen egg white	

6. MICROBIAL PHYSIOLOGY AND INTERACTIONS

Microbial physiology

Experiment 6.1: Growth curve of *E. coli* in liquid culture

The growth of unicellular microorganisms in a liquid culture medium is not linear but shows different growth phases. A so-called lag-phase in which the organism adapts to the growth conditions but does not yet divide, is followed by a phase of exponential growth (referred to as log-phase) and finally the cell density reaches a maximum where the cells stop to divide (stationary phase).

Aim:

The growth behaviour of unicellular microorganisms in liquid culture shall be demonstrated by photometric monitoring of the cell density of a growing *Escherichia coli* culture. The resulting growth curve allows the determination of the generation time of the bacterium. This experiment is also used to introduce and practice the liquid cultivation technique for bacteria.

Method:

The higher the cell density (optical density), the lower the light transmission of the liquid. The behaviour of such suspensions can be described in approximation by the law of Beer-Lambert, although this is in principle only valid for solutions. The concentration of the solution or the cell density of a suspension is thereby proportional to I_0/I. The negative logarithm of entering and leaving light intensity ($-\log I_0/I$) is called extinction or optical density (OD), respectively. Measurements are only meaningful up to 0.8, because at higher values the linear relationship between OD and concentration is not given any more. The OD is often measured at 600 nm (OD600). Unicellular microorganisms propagate by duplication. This is why the cell density of a growing culture is increasing exponentially and can be described with the following formula:

$$OD(t_2) = OD(t_1).e^{\mu.t}$$

t = time of measurement; μ = growth rate (min^{-1})

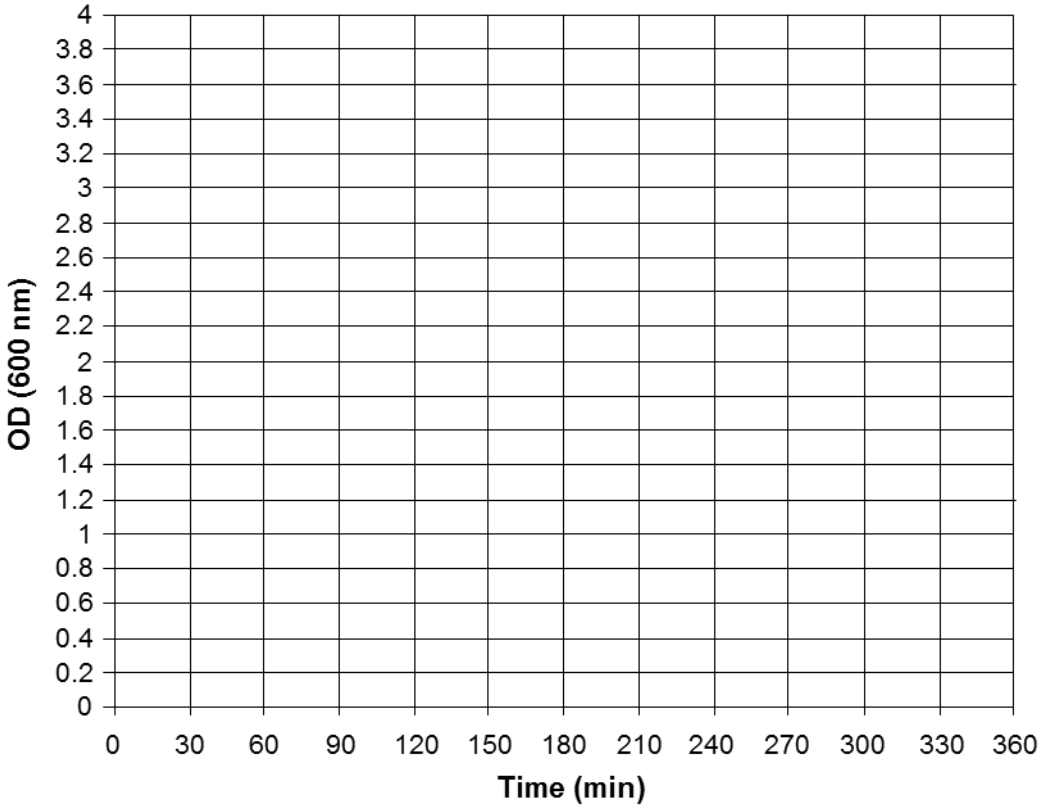
The growth rate is specific for a given organism and depends on the growth conditions (growth medium, temperature etc.). This is why it needs to be determined experimentally. A growth curve describes the change of the optical density (cell density) of a culture as a function of time and serves as basis for the determination of the growth rate and doubling (generation) time.

Material (per assistant group):

- *E. coli* overnight culture (*E. coli* BL21) in 5ml LBG
- LBG-liquid medium from Experiment 1.1
- 2 sterile 250 ml Erlenmeyer flasks with baffles

Procedure:

- Pipet 50 ml LBG-liquid medium from Experiment 1.1 into a sterile 250 ml Erlenmeyer flask.
- Inoculate the 50 ml liquid medium with 5 ml overnight culture (-> OD600nm = 0.1) and incubate on a shaking platform at 37°C.
- Remove 1 ml of diluted culture at the beginning and thereafter every 30 min for the determination of OD600nm. Sterile medium is used for blanking.
- Enter values in the coordinate system below
- Indicate the different growth phases (see above)
- From the values of the log phase, calculate the generation (doubling) time according to the formula $t_{1/2} = \ln 2/\mu =$ _____ min.



Experiment 6.2: Bacterial growth under aerobic and anaerobic conditions

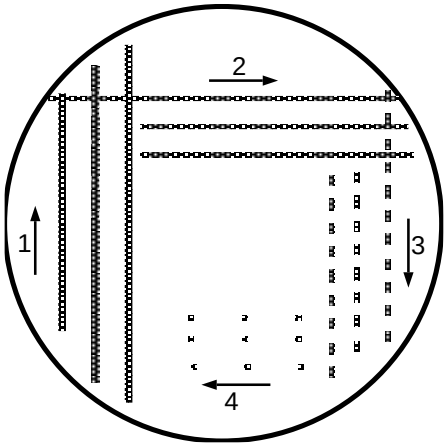
As mentioned in the introduction of Chapter 1, microorganisms are extremely versatile with regard to their metabolism e.g. in their oxygen requirement for energy production. Many bacteria are able to produce energy by other means than aerobic respiration with oxygen as terminal electron acceptor. In this experiment, the metabolic versatility of different bacteria shall be demonstrated on the example of growth on LBG agar plates under aerobic and anaerobic conditions.

Materials:

- Cultures of *Clostridium butyricum*, *Pseudomonas putida*, *Enterococcus faecalis* and *Micrococcus luteus* on plate
- 2 LBG agar (NA for *M. luteus*) plates per group of 2 students
- Anaerobic jars (1 per two assistant groups)

Procedure:

A small portion of bacteria material is taken from the main culture with an annealed and cooled off inoculating loop. A sterile agar plate is inoculated by diluting the bacteria across the agar surface according to the scheme below (dilution streaking, see Experiment 1.2 for detailed instructions). LBG agar (NA for *M. luteus*) plates are inoculated in this way with one of above mentioned bacteria (2 plates per organism, label the plates accordingly!).



One agar plate of each organism is incubated aerobically at room temperature without any further manipulation. The other agar plates are collected and placed in an anaerobic jar (one jar per two assistant groups). The jar is hermetically sealed and the air in the jar is replaced, using a gas station (assistants will show it), with an atmosphere consisting of 10% CO2, 7% H2 and 83% N2 at slight overpressure. The jar prepared in this way is then incubated at room temperature.

Characteristics of used bacteria:

Clostridium butyricum is a Gram-positive, obligate anaerobic, spore forming rod. This bacterium is very motile due to peritrichous flagella. *C. butyricum* is very common in nature. One of its metabolites is butyric acid which causes a distinctive repellent smell of the culture.

Enterococcus faecalis is a Gram-positive coccus which forms typical chains during the logarithmic growth phase. These bacteria are ubiquitous and are for example present in dairy products and other organic substances which are easily degradable.

Pseudomonas putida belongs to the bacteria of the genus *Pseudomonas* which are common in soil and in association with plants. They are short, mobile and Gram-negative rods.

Micrococcus luteus is a Gram-positive coccus that arranges in tetrads or irregular clusters during vegetative growth. The bacterium forms yellow colonies, belongs taxonomically (in contrast to *Enterococcus*) to the actinobacteria and can be found in many places such as the human skin, water, dust, and soil.

Evaluation:

Check the bacterial growth under aerobic and anaerobic conditions (+ growth; - no growth) based on the SIZE OF SINGLE COLONIES and decide on this basis whether the listed bacteria are of obligate anaerobic (oxygen is toxic for these organisms), obligate aerobic (need oxygen for growth) or facultative anaerobic (can grow under both conditions) type (collect the results for all bacteria and not just the ones that you tested!):

Bacterium	**with O_2**	**without O_2**	**Type**
Clostridium butyricum			
Enterococcus faecalis			
Pseudomonas putida			
Micrococcus luteus			

Experiment 6.3: Absorptive nutrition mode of microorganisms

Bacterial and fungal cells are surrounded by cell walls and produce exoenzymes converting larger molecules extracellularly to smaller molecules that can be taken up by the microbes via absorption (absorptive nutrition mode). Depending on their natural habitat, microorganisms differ in the spectrum of hydrolytic exoenzymes produced.

In the following experiment, a fungus and a bacterium are compared with regard to their production of proteolytic exoenzymes.

Material:

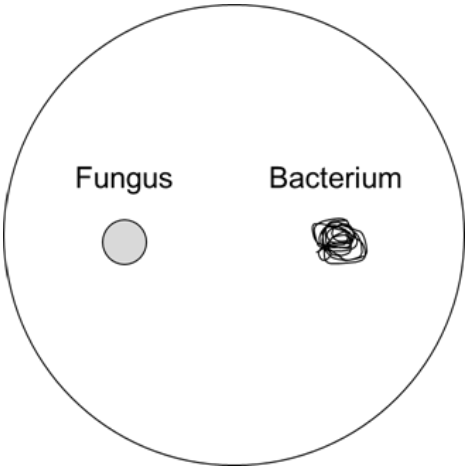
- 1 milk agar plate per group of 2 students
- 1 Petri dish culture of *Agaricus bisporus* (basidiomycete fungus, with pre-cut plugs) and *Bacillus subtilis* (Gram-positive bacterium) one per assistant group

Procedure:

The bacterium is streaked and the plugs with the fungal mycelium (upside down orientation of the plug i.e. fungal mycelium faces agar surface) are placed onto the milk agar plate according to the scheme below. DO NOT invert plates after inoculation since the plug with the fungal mycelium could fall off. Incubate at room temperature overnight and at 4°C for 6 days to promote bacterial growth and avoid fungal growth (could interfere with the evaluation of the experiment since fungal colony covers halo around plug).

Evaluation:

Milk protein is visible as turbidity of the plates. A clearing zone around the fungal and the bacterial colonies indicate the secretion of proteases. Indicate the observed clearing zones in the scheme below.



Experiment 6.4: Fungal spores: Distribution of spores, phototropism of sporophors and light regulation of spore development

Although fungi are chemoorganotrophic and are not able to perform photosynthesis, they have light receptors which regulate e.g. the spore development (phototropism and light regulation of development). Fungi are also able to "sense" gravity and orient their spore-producing structures accordingly (gravitropism). The zygomycete *Phycomyces blakesleeanus* is a well-known model organism for the physiological impact by light and gravity on fungi.

Material (per group of 2 students):

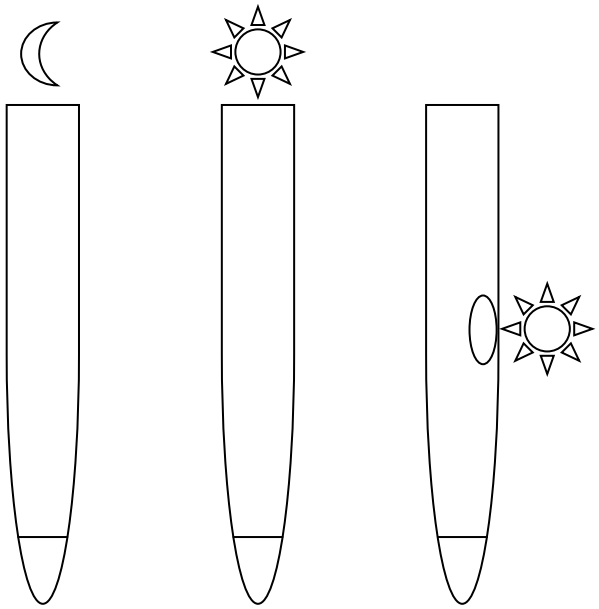
- 1 slope agar tube containing sporulating *Phycomyces blakesleeanus* (B)
- 3 slope agar tubes containing 3 ml CMA-agar
- Aluminum foil
- Plastic rack with large holes for incubation of the aluminium-wrapped slope agar tubes

Procedure:

- Add 1 ml sterile water to the slope agar culture and vortex thoroughly.
- Decant the spore suspension into a sterile Eppendorf tube.
- Inoculate each CMA-slope agar tube with 0.1 ml spore suspension.
- Wrap one reaction tube with aluminium foil until only a 5 mm window is left at the top of the tube.
- The other two tubes are completely wrapped with aluminium foil (including the cap). In one tube, a small window in the middle of the tube (diameter app. 5 mm) is cut out.
- The tubes are incubated vertically (to avoid gravity effects) for one week at room temperature on the bench.

Evaluation:

Unwrap the tubes and compare the morphology of the fungus grown at the various light conditions. Draw the observed morphology of the fungus into the scheme below (the moon stands for darkness, the sun for light). What are the features pointing to phototropism and light-dependent development of the fungus?



Experiment 6.5: Horizontal gene transfer between *E. coli* strains by conjugation

The transfer of genetic information to offspring during reproduction of an organism is called vertical gene transfer ("inheritance"). Eukaryotic organisms like fungi (see chapter 4) – recombine their whole genome during sexual reproduction and the individuals are characterised by their great genetic variability. Prokaryotic organisms like bacteria reproduce solely by mitotic cell division (asexual reproduction) and pass their genetic information unchanged on to the next generation.

In addition to the vertical gene transfer, the genetic information can be exchanged between individuals of the same generation of a specific species or even between individuals of different species (horizontal gene transfer). With bacteria, three different transfer mechanisms of horizontal gene transfer can be distinguished: conjugation, transformation (see P GL Bio II, Molecular Biology Part) and transduction. With all these mechanisms, the transferred DNA has to be replicated within the recipient cell e.g. as an episomal genetic element (plasmid) with its own replication origin or by recombination into the host chromosome in order to ensure inheritance of the encoded characters by vertical gene transfer.

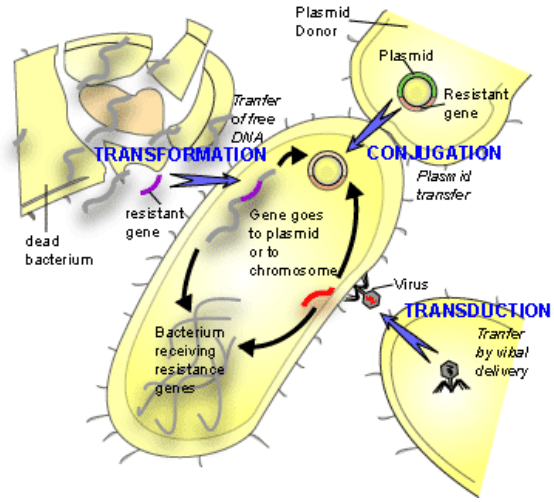


Figure 6.1: Schematic representation of different routes of horizontal gene transfer between bacteria. See text for details.

Gene transfer by conjugation has been discovered with *Escherichia coli* and is often encountered among Enterobacteriaceae. The ability for gene transfer depends on an extrachromosomal element (plasmid), the so-called fertility (F)-plasmid. The F-plasmid contains the genes necessary for the conjugation process e.g. the genes coding for the F-pili, surface structures by which the gene transfer takes place (see Fig. 6.2, left panel). Normally, only the F-plasmid and no chromosomal DNA is transferred. Transfer of chromosomal DNA is a consequence of the insertion of the F-plasmid into the bacterial chromosome. During conjugation of such „high frequency of recombination"(Hfr)-cells, transferred chromosomal DNA of the donor cell is recombined with high frequency with the chromosomal DNA of the receiving cell. The transfer of the chromosomal DNA occurs as "rolling circle" and is determined by the site and the orientation of the integrated F-plasmid (H, P801, P4X etc. in Fig. 6.1, right panel). The transfer is initiated at the insertion site of the F-plasmid and proceeds in the opposite direction of its orientation i.e. in the case of HfrH the *thr*- and *leu*-biosynthesis genes are transferred very early in the conjugation process. Since the speed of DNA transfer is constant, genes can be mapped by the time of entry into the receiving cell. The transfer of the complete *E. coli* chromosome takes app. 100 min. (see Fig. 6.2, right panel). The *thr*- and *leu*-biosynthesis genes in HfrH are transferred within the first 10 min. of conjugation.

Aim:

The transfer and recombination of chromosomal DNA by conjugation is demonstrated by the using the *E. coli* Hfr donor strain CGSC#259 (HfrH) and the recipient strain CGSC#7541 (F^- *thr-1 leuB*6 StrR).

NOTE: leuB6 and *thr-1* indicate mutations causing auxotrophies of the respective strains for thiamin, leucine and threonine; StrR indicates a resistance of the respective strain towards streptomycin.

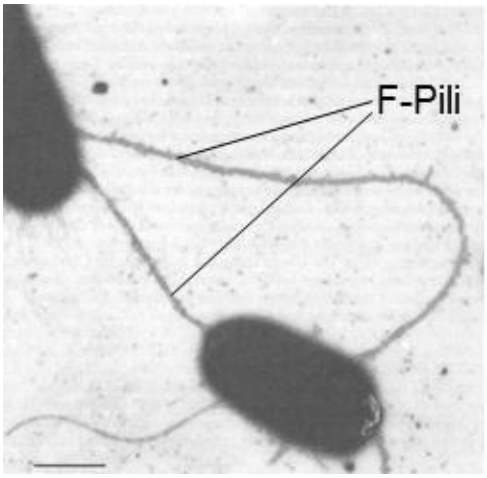
Material:

Per group of 2 students:

- Fresh overnight cultures of both *E. coli* strains in LBG complete medium
- 2 reaction tubes with 5 ml LBG medium each
- 4 *E. coli*-minimal medium (MME) plates containing 50 mg/l streptomycin but no threonine or leucine
- Petri dishes with ethanol and Drigalski spatula for plating

Procedure:

1. 100 µl of the overnight culture of each strain is 50fold diluted into 5 ml LBG-medium and shaken for 2.5 h at 37°C to reach the exponential growth phase.
2. 100 µl of the Hfr-strain (CGSC#259; donor) is mixed with 900 µl of the F^--strain (CGSC#7541; recipient) and incubated without shaking for 30 min. at 37°C.
3. 100 µl of the mixture as well as 100 µl each of the donor and the recipient exponential cultures are spun down (5 min. at max. rpm) in sterile Eppendorf tubes (3 separate tubes).
4. The cells are resuspended in 250 µl of sterile water by vortexing.
5. A 1:10 dilution of the mixture in 250 µl of sterile water is prepared und plated entirely on a MME Str plate using the Drigalski spatula. Afterwards, the three UNDILUTED bacterial suspensions (donor, recipient and mixture) are plated as well. *NOTE:* Due to the 25 µl used for the dilution, only 225 µl of the undiluted mixture are available for plating.
6. The plates are incubated over the weekend at 30°C.



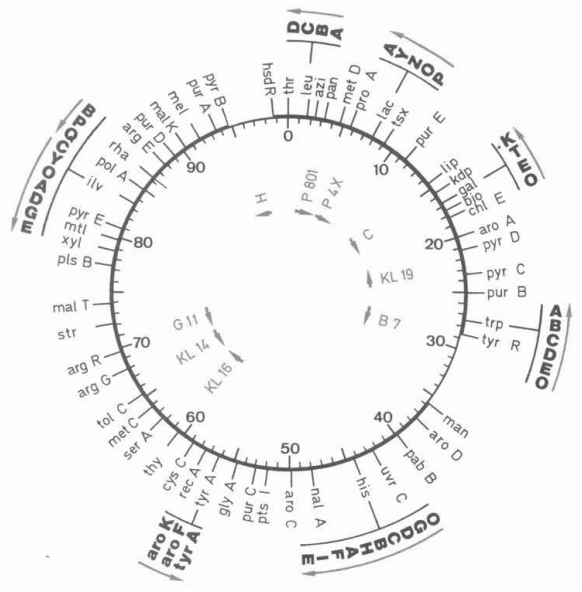


Figure 6.2: Conjugation of *E. coli* (from Schlegel, Allg. Mikrobiologie) Left: Electron micrograph of conjugating *E. coli*-cells with F-pili. Right: Genomic map of the circular *E. coli*-chromosome (Units: min.) with site of integration and orientation of the F-plasmid in various Hfr-strains (labeled arrows within the circle). The F-plasmid in HfrH-strains (indicated by H) is integrated at around 0 min and points from right to left, so the chromosomal genes of this strain are

transferred, starting at around 0 min., in direction from left to right i.e. from the *thr* genes to the *leu* genes.

Results:

Enter obtained number of colonies on MME Str for originating strains and conjugation mixture in the table below:

Strain	**Colonies per 100 µl**
CGSC#259	
CGSC#7541	
CGSC#259 x 7541	

Experiment 6.6: Transcriptional regulation of the key enzyme of microbial nitrogen fixation

Biological N_2-fixation takes an important position in the natural nitrogen cycle. It compensates losses which occur through denitrification, washing out of nitrate or NH_3-diffusion from the soil. It is estimated, that 100 - 200 x 10^6 tons of nitrogen worldwide are brought back to the soil by biological N_2-fixation. Only prokaryotic microorganisms possess this metabolic pathway. In Tables 6.1 and 6.2, a selection of N_2-fixing bacteria is listed. It shows that the N_2-fixation is found in bacteria of diverse systematic groups. The list contains for example Gram-positive as well as Gram-negative bacteria, strictly aerobic, facultative and strict anaerobic bacteria, photosynthetic and autotrophic bacteria and free-living and symbiotic bacteria.

Tables 6.1 and 6.2: A selection of nitrogen fixing bacteria

Free-living bacteria	
Aerobic-micro-aerobic	
Azotobacter vinelandii	*Azospirillum brasilense*
Anabaena variabilis	*Methylococcus caspsulatus*
Xanthobacter autotrophicum	*Bacillus polymyxa*
Facultative anaerobic	
Erwinia herbicola	*Rhodopseudomonas capsulata*
Klebsiella pneumoniae	*Rhodospirillum rubrum*
Obligatory anaerobic	
Clostridium pasteurianum	*Desulfotomaculum ruminis*
Chromatium vinosum	*Methanogenic bacteria*
Chlorobium limicola	

Symbiotic bacteria	Symbiosis partner
Rhizobium trifolii	Clover
Rhizobium meliloti	Lucerne
Rhizobium leguminosarum	Pea
Rhizobium phaseoli	Bean
Bradyrhizobium japonicum	Soybean
Bradyrhizobium sp. (Lupinus)	Lupine
Frankia sp. (Alnus)	Alder
Anabaena azollae	Azolla (fern)

Genetics and regulation

The analysis of the genes important for the nitrogen fixation (nif genes) has been elucidated over the past decades. According to the latest in vivo research it is estimated that the N_2-fixing cell hydrolyses 20 to 25 molecules of ATP in order to maintain the reduction of one molecule of N_2. This enormous energy consumption as well as the biochemical and genetic complexity of the nitrogenase system are the price the cell has to pay to break the inert triple bond of nitrogen. Therefore it is understandable that nitrogen fixing bacteria have regulation mechanisms that allow them to stop the N_2-fixation immediately, if there is no need for it.

For the most part, regulation takes place on the genetic level, i.e. the expression of the nif genes is regulated. Two genetic control mechanisms are especially important for it:

1. **N-control:** The nif-expression is blocked if the cell is offered a competing, more “economic” nitrogen source such as ammonium, nitrate, urea or amino acids.

2. **Redox control:** Oxygen is not only toxic for the nitrogenase complex, but also represses the expression of the nif genes very effectively via the redox state of the cell.

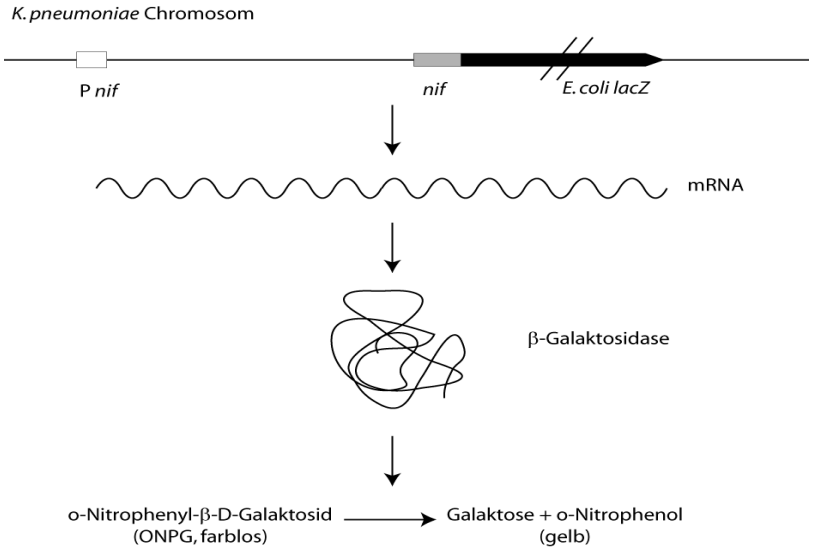
Klebsiella pneumoniae is a free-living, Gram-negative, facultative anaerobic bacterium belonging to the family of *Enterobacteriaceae*. The *nif* genes of *K. pneumoniae* are derepressed only under the following conditions:

- Growth under anaerobic conditions
- Medium poor in nitrogen
- Temperatures below 30 °C

A simple method to monitor the expression of the *nif* genes is the use of so-called *nif::lacZ*-fusions (Fig. 6.3). The β-galactosidase coding *lacZ* gene is inserted into a *K. pneumoniae nif* gene in such a way that it is expressed under the control of the *nif* promoter (P) as are the other *nif* genes. The enzyme β-galactosidase cleaves the colourless *o*-nitronphenyl-β-D-

galactoside in galactose and *o*-Nitrophnol (yellow). The yellow coloration is a measure for the amount of β-galactosidase and thus also a measure for the activity of the *nif* promoter.

Figure 6.3: Principle of assaying the transcriptional/translational activity of a *nif* gene using a *lacZ* fusion



In the following experiment, the derepression of the *nif* genes shall be monitored under various growth conditions by assaying the β-galactosidase-activity of a strain carrying a *nif::lacZ* fusion (*K. pneumoniae* UNF744). In a well aerated culture, the *nif* genes are switched off, whereas they are active (derepressed) in an anaerobic culture. High concentrations of a nitrogen source in the medium, such as glutamine, inhibit derepression.

Procedure

Material:

- NFDM-medium (without Gln, as a reference for OD600nm measurement)
- Z-buffer
- Chloroform
- 0.1% SDS (Sodium-Dodecyl-Sulfate)
- ONPG-solution (4 mg/ml) in 0.1 M Phosphat-buffer pH 7.0
- 1 M Na_2CO_3
- Glass reaction tubes, plastic cuvettes
- Spectrophotometer

NFDM Minimalmedium pH 7.0 (Nitrogen free derepression medium)

K_2HPO_4	12.06 g/l
KH_2PO_4	3.4 g/l
Glucose	20.0 g/l

Casamino acids	0.2 g/l
Yeast extract	0.2 g/l
$MgSO_4 \cdot 7H_2O$	0.1 g/l
$FeSO_4 \cdot 7H_2O$	0.033 g/l
$Na_2MoO_4 \cdot 2H_2O$	0.025 g/l

Z-buffer pH 7.0

Na_2HPO_4	8.5 g/l
NaH_2PO_4 x $2H_2O$	6.2 g/l
KCl	0.75 g/l
$MgSO_4 \cdot 7H_2O$	0.246 g/l
β-Mercapotethanol	2.7 ml/l (added after autoclaving before use)

Preparation (by organizer):

Cultivation of *K. pneumoniae* under various conditions:

#1:	aerobically (Erlenmeyerflask)	200 µg/ml Glutamin	(yellow)
#2:	anaerobically (Falcon tube)	200 µg/ml Glutamin	(green)
#3:	anaerobically (Falcon tube)	500 µg/ml Glutamin	(blue)
#4:	anaerobically (Falcon tube)	2000 µg/ml Glutamin	(red)

All cultures were incubated overnight at 30 °C. The Erlenmeyer flask was shaken with 160 U/min and therefore well aerated. The Falcon tubes were only slightly shaken to avoid sedimentation of the cells.

NOTE: Each group of 2 students will measure all 4 cultures and compare their absolute and relative numerical values with the other groups in the assistant group.

Determination of the optical density:

- Transfer 250 µl from each culture into a cuvette which contains already 750 µl NFDM (minimal medium). In addition, prepare a cuvette containing solely 1 ml NFDM medium.
- Determine the optical density at 600 nm (OD600) using the spectrophotometer; it is a measure for the cell density of the cultures. Use the cuvette containing solely NFDM medium as a blank.
- Enter the OD600 in the table below (NOTE: consider the dilution in your calculations)

β-Galactosidase-assay

- Prepare **5 glasstubes** per group of 2 students, add **0.8 ml of Z-buffer** each and label them with 1, 2, 3, and 4 according to above numbers of the **different cultures** and 0 as **negative control**.

- Add **0.2 ml of the respective cultures** into tubes 1-4. Add **0.2 ml NFDM** to the negative control (tube 0).
- Add **50 µl of 0.1 % SDS** and **100 µl of chloroform** to all tubes to permeabilize the cells
  (NOTE: Chloroform is volatile. Equilibrate the atmosphere in the Gilson pipette with chloroform by pipetting chloroform up and down 2-3 times before transferring the 100 µl. Add the chloroform directly into the suspension to avoid evaporation)
- **Vortex** the tubes for 5 sec each; the suspension should become turbid.
- Pre-incubate the tubes for **5 min at 28 °C** in the water bath to reach the reaction temperature.
- Add **0.2 ml of ONPG-solution** in the first tube to start the reaction and every 30 s for the following ones, vortex and place back into the water bath (NOTE: Start a timer or note the time)
- After app. 10-15 min, the reaction is stopped in all tubes in the same rhythm as for the start by adding **0.5 ml of 1 M Na_2CO_3** und vortexing. (NOTE: Stop the timer or note the time)
- Pour the content of the tubes carefully into an **Eppendorf tube**. Centrifuge the tubes for **2 min at 10'000 rpm** and transfer 0.9 ml of the supernatant carefully into a **cuvette**.
- **Determine the OD420 of the reactions 1-4** using reaction 0 as blank. Enter the data into the table below.
- **Calculate** the activity of the β-galactosidase according to below formula:

$$U = (OD_{420} \times 1000) : (V \times dt \times OD_{600})$$

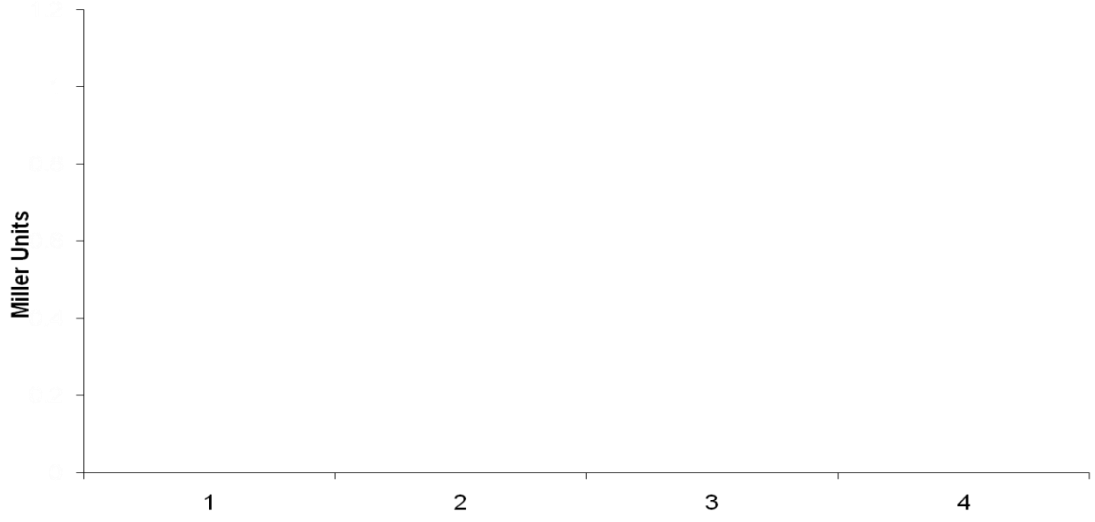
OD420 Absorption at 420 nm of the reaction at the end
OD600 Absorption at 600 nm of the culture used for the reaction
U: Enzymatic activity [Unit]
V: Culture volume that was used in the reaction [ml]
dt: Reaction time [min]

- **Enter** the results in below table (under 'a') and draw a histogram for better visualization

		Gln µg/ml	OD600	OD420	dt [min]	Activity U
1a	aerobic	200				
1b	aerobic	200				
1c	aerobic	200				
1d	aerobic	200				
2a	anaerobic	200				
2b	anaerobic	200				
2c	anaerobic	200				
2d	anaerobic	200				
3a	anaerobic	500				
3b	anaerobic	500				
3c	anaerobic	500				
3d	anaerobic	500				
4a	anaerobic	2000				
4b	anaerobic	2000				
4c	anaerobic	2000				
4d	anaerobic	2000				

a, b, c, d: Different student groups within assistant group (with 'a' being own group)

Visualize the results graphically as a histogram (column representation):



- **Compare** your results with the other student groups in your assistant group and enter them in your table. Discuss eventual differences and experimental pitfalls possibly explaining the differences. From these discussions: Can you imagine the meaning of **'technical' and 'biological' replicates** to yield significant data?

Microbial interactions

Experiment 6.7: Quorum sensing by bacteria

Gram-negative and Gram-positive bacteria of the same genus and even from different genera can communicate with each other via chemical messengers. This type of communication is called "quorum sensing" since the response of the bacteria to the messengers depends on the concentration of the messenger in the medium which correlates to the number of messenger-secreting bacteria ('quorum'). Examples of bacterial phenomena, which are regulated by quorum sensing, are: bioluminescence, virulence, conjugation, transfer of mobile DNA, antibiotics-synthesis or biofilm formation. Since the pathogenicity of bacteria is often coupled with biofilm formation (which makes the treatment with antibiotics less effective), a combinatorial treatment of bacterial infections with antibiotics and agents that interfere with quorum sensing (e.g. from garlic) of the pathogenic bacteria is discussed.

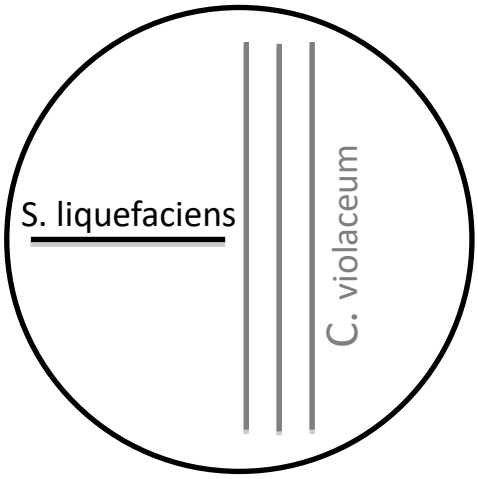
In a simple experiment we will demonstrate the communication between two different genera of Gram-negative bacteria, *Serratia liquefaciens* and *Chromobacterium vilaceum*, which speak the same chemical language. The communication between the two bacteria is visualized by the quorum sensing-dependent production of a violet pigment (violacein) in *C. violaceum*. The *C. violaceum* used for the experiment itself is defective in its own production of the quorum sensing messenger AHL (acyl-homoserine-lactone).

Materials per student group:

- 1 LBG agar plate
- Plate with *S. liquefaciens*
- Plate with *C. violaceum*

Procedure:

Streak out *C. violaceum* cells in 3 lines close to each other as indicated in the scheme below. Then streak out *S. liquefaciens* in a 90° angle to the *C. violaceum* streaks so they almost touch each other. Incubate the cells at RT for several days.



Evaluation:

Indicate the region of the blue colour in the scheme above.

Experiment 6.8: Isolation of a major human commensal and biofilm forming bacterium from the oral cavity

The term biofilm describes a community of microorganisms of the same or different kind that is encased by a layer of self-produced matrix (slime). A biofilm arises when microorganisms colonize a solid or liquid surface. If the environment is humid and there is sufficient supply with nutrients, the microorganisms secrete certain sugars and other substances that form a slimy layer termed extracellular polymeric substance (EPS). The constituents of the EPS are polysaccharides, proteins, glycoproteins and other hydrophilic polymeric substances. Examples are the dental plaque or the slimy layers in flower vases, river pebbles or water pipes.

In a biofilm, bacteria live in a kind of organized community, in which they communicate with each other (see Experiment 6.7) and differentiate to cells with specialized functions similar to a multicellular organism. Biofilm formation prevents bacteria from being flushed away from sites of constant but flowing nutrient supply (oral cavity, intestine, river etc.) and protects them from environmental influences such as extreme temperatures or pH values. In medicine, biofilms are of special importance: bacteria, that are organized in biofilms, are well protected from our immune system and from antibiotics. Biofilms can be up to 1000fold more resistant against antibiotics than disperse (planktonic) forms of the same bacterium.

Streptococcus salivarius is a gram-positive lactic acid bacterium that ferments carbohydrates (glucose) and produces lactic acid. It is one of the first bacteria colonizing the oral mucosa after birth and is maintained as a dominant population in the human oral cavity and the upper airways throughout the life of its human carrier. The bacterium is also part of the human

intestinal microflora and has been isolated from fecal samples. Several reports indicate that *S. salivarius* plays a positive role in human oral and digestive tract ecology. *S. salivarius* may exert its impact on human health through effects on the stability of microbiota composition, bacterial interference and interaction with the host.

Figure 6.4: A probiotic containing S. salivarius claimed to maintain a healthy oral bacterial flora. Original description: *'Solaray® Oral Flora™ lozenges are intended to provide nutritive support for normal, healthy mouth and throat flora. Streptococcus salivarius K12 is a friendly, naturally-occurring oral bacteria intended to help return the mouth and throat to a balanced state for fresh breath'*.

In this experiment, the ability of *S. salivarius* to grow on media with a very high concentration (50 g/l, see Addendum) of saccharose is exploited to isolate the bacterium from human oral mucosa. On such saccharose-containing plates, *S. salivarius* forms characteristic slimy colonies that do not spread but pile up during growth - eventually to the lid of the petri dish. In this regard, these colonies are simple examples of bacterial biofilms.

Material:

Sterile cotton swabs, saccharose-agar (SCA) plates, plates with pure cultures of *E. coli* (from Experiment 2.3) and *S. salivarius*.

Procedure:

Samples of the oral mucosa are spread on saccharose-agar plates and LBG-plates (as control) (1 plate of each per student). The plates are sealed with parafilm and incubated for 2-3 weeks at room temperature to isolate bacterial colonies from the mouth mucosa with characteristic formation of slime. Pure cultures of *E. coli* (negative control) and *S. salivarius* (positive control) are used as controls for slime formation. For better comparison, these strains are streaked next to each other on a saccharose-agar plate (1 plate per student group).

Evaluation:

Are there differences between the various samples (mucosa, controls) with regard to the morphologies of the single colonies? Which samples yield slime-forming colonies?

ANSWER:

Experiment 6.9: The phyllosphere as a niche for methylotrophic bacteria

With regard to colonization of terrestrial plants by microorganisms one often distinguishes between the underground part of the plant, the rhizosphere, and the aboveground part of the plant, the phyllosphere. The estimated worldwide leaf surface of 6.4×10^8 km^2 represents a huge habitat for microorganisms. For comparison: the surface of all the continents comprises only 1.5×10^8 km^2. The leaf surfaces of plants are colonized by approximately 10^6 to 10^7 bacterial cells/cm^2. The leaf is colonized not only by epiphytic microorganisms on its surface but also by endophytes that live within the inner part of the leaf, the apoplast, and can penetrate even to the xylem. The most frequent colonizers of the phyllosphere are representatives of Gram-negative proteobacteria including the genera *Pseudomonas* and *Methylobacterium*. However, also Gram-positive bacteria such as *Bacillus* spp.are found. Furthermore, leaves are colonized by filamentous fungi, yeasts, algae and sometimes by protozoa and nematodes but in lower abundance.

The genus *Methylobacterium*

Characteristics of the genus *Methylobacterium* are the pink pigmentation (carotinoids) and the facultative methylotrophic metabolism. The term “methylotroph” describes the ability to grow on substrates without C-C bonds. Methylotrophic microorganisms are able to synthesize their cellular components from and gain the required energy by oxidation of reduced C1-compounds. Utilizable C1-compounds are methanol (CH_3OH), formaldehyde (CH_2O) and formic acid (HCOOH); also methylamines (e.g. CH_3-NH_2), methylated sulphur compounds (e.g. CH_3-SH) or halogenated methane compounds (e.g. CH_3-Cl) are utilized by representatives of the genus *Methylobacterium*. The ability to grow on C1-compounds requires a special metabolism. The energy necessary for survival and growth is gained from the stepwise oxidation of the reduced substrate to CO_2 (see Fig. 6.5). This process produces reducing equivalents which can be transformed into ATP by the respiratory chain. The biosynthesis of the cellular components of methylotrophic organisms is based exclusively on C1-building blocks. Representatives of the genus *Methylobacterium* utilize for this purpose the toxic formaldehyde and CO_2, which are assimilated via the serine pathway (Fig. 6.5). Facultative methylotrophy allows methylobacteria to utilize, besides C1-compounds, also more complex substrates such as succinate or pyruvate as carbon and energy source if they are available.

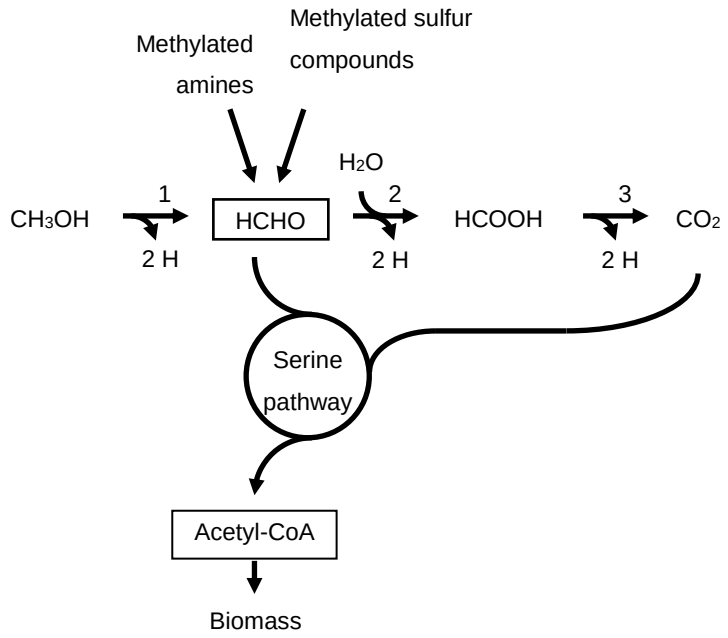


Figure 6.5: C_1-Metabolism in *Methylobacterium*. 1. Methanol dehydrogenase, 2. Formaldehyde oxidation system, 3. Formiat dehydrogenase (modified from Lidstrom, The Prokaryotes, 2006)

There are additional methylotrophic microorganismes besides the genus *Methylobacterium* some of which are obligate methylotrophs e.g. the genera *Methylobacillus* and *Methylophilus*; as well as the subgroup of methane-utilizing bacteria, the so-called methanotrophs, amongst e.g. *Methylococcus*. Additional facultative methylotrophic organisms are found within the genera *Hyphomicrobium*, *Paracoccus* and *Bacillus*. It should be mentioned that there are, besides methylotrophic bacteria, methylotrophic yeasts e.g. within the genera *Hansenula, Pichia* oder *Candida*.

Methylotrophic bacteria are widely distributed in nature. They are found in diverse aquatic and terrestrial habitats. They are however also present in dust and air. Therefore it is not surprising that representatives of the genus *Methylobacterium* were found as contamination in a space shuttle and that the pinkish shiny slime on shower curtains also consists of *Methylobacterium*. The presence of *Methylobacterium* was also shown in human mouth and on human skin.

The consistent colonization of plants by representatives of the genus *Methylobacterium* is remarkable. This genus was found on almost every plant examined. *Methylobacterium* colonizes in particular the phyllosphere. These bacteria are found on leaf surfaces and in leaves, in the apoplast. In the meantime, it is known that methylobacteria can profit from methanol that is produced and emitted by the plant. The plant produces methanol as a side product during cell wall biosynthesis. However, facultative methylotrophy allows

methylobacteria to utilize also other carbon sources which are available on the leaf surface. The ability to utilize methanol is however of advantage for the methylobacteria in the competition with other leaf colonizers for carbon sources.

The preparation of leafprints is a simple and rapid procedure to determine the presence and the location of microorganisms on leaves. Aim of the experiment is to determine the presence and location of *Methylobacterium* on white clover (*Trifolium repens*) or any other plant leaf. In order to select for the growth of Methylobacterium, the following minimal medium with methanol as sole carbon source (MMM) is used:

Composition		*Trace element solution*	
$(NH_4)_2SO_4$	2.0 g/l	EDTA	1.50 g/l
$MgSO_4 \times 7\ H_2O$	0.2 g/l	$ZnSO_4 \times 7\ H_2O$	4.40 g/l
$NaH_2PO_4 \times 2\ H_2O$	1.7 g/l	$CaCl_2 \times 2\ H_2O$	1.47 g/l
K_2HPO_4	1.7 g/l	$MnSO_4 \times 4\ H_2O$	0.10 g/l
Trace element solution	1 ml/l	$FeSO_4 \times 7\ H_2O$	1.00 g/l
Agar	15 g/l	$(NH_4)Mo_7O_{24} \times 4\ H_2O$	0.22 g/l
pH 7.0		$CuSO_4 \times 5\ H_2O$	0.30 g/l
		$CoCl_2 \times 6\ H_2O$	0.30 g/l

After autoclaving 5 ml/l sterile filtered methanol are added to the medium.

Material (per group of students):

- LBG-agar plate with *E. coli*
- 1 MMM agar plate
- 1 LBG agar plate (control for selection on MMM)
- 2 trifoliates of white clover or other plant leafs
- Sterile petri dish for the transport of the trifoliates/plant leaves

Procedure:

The trifoliates are harvested without contamination, i.e. you should wear gloves and you should touch only the stipes of the trifoliates. The trifoliates are transferred to the laboratory in a petri dish. The leaf prints are prepared with the lower side of the leaves since this side is colonized more frequently than the upper side. The reason for this is that the growth conditions on the lower side of the leaf are less stringent than on the upper side (UV-irradiation, dryness). For the preparation of the leaf print, the trifoliate is placed carefully in the middle of the agar plate. Use your fingers to press the single leaves of the trifoliate onto the agar in order to ensure sufficient contact with the agar surface. Watch out that the leaves are not shifted during this procedure and that the fingers do not touch the agar. Afterwards, you remove the trifoliate from the plate.

One trifoliate is used for the leaf print on MMM and the other for the leaf print on LBG. The plates are incubated at 30°C for 1 - 2 days (LBG plates) or 5 - 7 days (MMM plates). As selectivity-control *E. coli* is streaked out on both plates next to the leaf replicas.

Evaluation:

a) Selectivity of the plates: How many different colony types are discernible on the different plates?

b) Localization of the methylobacteria on the MMM plates: Can you conclude anything about which locations of the leaf are preferred by the bacteria?

c) Which characteristic features of the genus *Methylobacterium* are exploited for enrichment and identification of this group of organisms?

7. ADDENDUM

Recipes for the media

H-Agar (H)

Tryptone	10	g/l
NaCl	5	g/l
Agar-Agar	15	g/l

Soft agar (H-Top Agar)

Tryptone	10	g/l
NaCl	5	g/l
Agar-Agar	6.5	g/l

Yeast complete medium (YPD)

Yeast extract	10	g/l
Peptone	20	g/l
Dextrose	20	g/l
Agar-Agar	15	g/l

Yeast-sporulation medium (HSpo)

Potassium acetate	10	g/l
Yeast extract	1	g/l
Dextrose	0.5	g/l
Agar-Agar	15	g/l

Milk agar (LA)

Rapilait powder	20	g/l
Peptone	5	g/l
MgSO4	0.25	g/l
KH2PO4	0.5	g/l
Agar-Agar	15	g/l

Starch agar (SA; for fungi)

Soluble starch	2	g/l
Peptone	10	g/l
MgSO4	0.25	g/l
KH2PO4	0.5	g/l
Agar-Agar	15	g/l

Luria Bertani Glucose (LBG) medium

Tryptone	10	g/l
Yeast extract	5	g/l
Glucose	5	g/l
NaCl	5	g/l
Agar-Agar	15	g/l
pH 7.5		

Tryptone Bile Glucuronid Agar (TBX)

Bile salts, purified	1.5	g/l
5-Bromine-4-chlorine-3-indolyl-	0.0	g/l
□-D-glucuronic acid Cyclohexyl-	75	
ammonium salt		
Peptone	20	g/l
Agar-Agar	15	g/l

Yeast Minimal medium (MV):

Yeast Nitrogen Base w/o amino	6.7	g/l
acids		
Dextrose	20	g/l
Agar-Agar	15	g/l
pH 6.0		

Corn meal agar (CMA)

Corn meal agar (Difco)	8.5	g/l
Malt extract	5	g/l
10% KOH	2	ml/l
Agar-Agar	7.5	g/l

Malt agar (MA)

Malt extract	30	g/l
Agar-Agar	15	g/l

Sporulation medium for Bacillus (BSpo)

Dextrose	3.0	g/l
Yeast extract	2.0	g/l
(NH4)2SO4	2.0	g/l
K2HPO4 . 3H2O	0.5	g/l
MgSO4 . 7H2O	0.2	g/l
MnSO4 . 4H2O	0.05	g/l
CaCl2 . 2H2O	0.08	g/l
pH 7.3		

Lactic acid (MS)-Agar

Tryptone	10	g/l
Yeast extract	5	g/l
Dextrose	10	g/l
K2HPO4	2	g/l
CaCO3-	3	g/l
Agar-Agar	15	g/l

Minimal medium (MME) for *E.coli*

MgSO4.7H2O	0.2	g/l
NaCitrate.2H2O	2	g/l
K2PO4	10	g/l
NaNH4HPO4.4H2O	3.5	g/l
Dextrose	5	g/l
Agar-Agar	15	g/l
pH 7.5		

Saccharose (SCA)-Agar

Saccharose (Sucrose)	50	g/l
Yeast extract	5	g/l
Tryptone	10	g/l
Gelatine	10	g/l
K2HPO4	3	g/l
Agar-Agar	20	g/l
pH 7.2		