Experiment	**Pathogenic Staphylococci of the Nose ?** **Molecular Diagnostics and Importance of Antibiotic Resistances**
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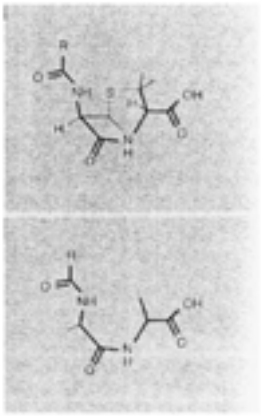
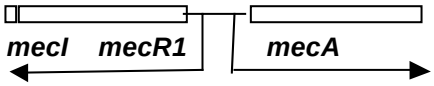
Reading	Chapters in BBOM 9th: **3.7, 18.7, 18.8, 18.12**, 19.2, 21.1, **21.3**, , 22.7, 22.8 in BBOM 9th: Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, Prentice Hall, 1999. ISBN: 0-13-085264-3
Objectives	To determine **antibiotic resistance** in an important human pathogen. To demonstrate the induction of the **penicillin-binding protein PBP2a** and **methicillin resistance**.
Background	*Staphylococcus aureus* is a normal, permanent or transient inhabitant of skin and mucous membranes in 10 to 30 % of healthy people. *S. aureus* has though, a pathogenic potential, and can produce **toxins** and **virulence factors**, causing superficial infections, food poisoning, and more serious diseases, such as endocarditis, osteomyelitis, bacteriaemia, and toxic shock. Staphylococci have the ability to develop relatively easily **resistance to antibiotics**, either by **mutation** or by **acquisition of resistance determinants**. Especially methicillin-resistant staphylococci are feared, because, besides their intrinsic resistance to all **beta-lactams** and derivatives, they have the tendency to acquire resistances to multiple additional, not related antibiotics. Due to the high antibiotic use and pressure in hospitals, some strains of *S. aureus* have become resistant to almost all commonly used antibiotics. It is therefore of importance to determine if an *S. aureus* isolate from a patient carries the methicillin resistance determinant, in order to take special precautions to prevent the spread of a potential methicillin-resistant *S. aureus* to other patients. Methicillin was the first **semi synthetic penicillinase-resistant beta-lactam**. Staphylococci that are resistant to methicillin are also resistant to penicillin and to all other penicillinase-resistant derivatives of beta-lactams, including cephalosporins and carbapenems. The **resistance is intrinsic**; this means that the antibiotic is not destroyed, but that it has no effect any more on the methicillin-resistant *S. aureus*. Resistance is due to the acquisition of the ***mecA* gene**, which codes for an additional penicillin-binding protein, called PBP2a. This PBP2a has a much lower affinity to beta-lactams than the cells own PBPs, and still functions at concentrations of methicillin, which inactivate the normal PBPs. PBPs catalyze the crosslinking of the peptide moiety of the **peptidoglycan**. Beta-lactams inhibit the **transpeptidase activity** of the PBPs and thus the crosslinking of the cell wall. This is lethal for the cells, because a strong cell wall is needed to maintain the shape of the bacteria and to confer strength against the internal osmotic pressure.



Penicillin mimicks the structure of the terminal D-Ala-D-Ala of the peptidoglycan precursor, the substrate of the PBPs. Penicillin binds to the active site of the transpeptidase domain of the PBPs and inactivates their function.

From: http://www.systbot.uni-tuebingen.de//penicillin/chemie.htmls

The synthesis of PBP2a is regulated:



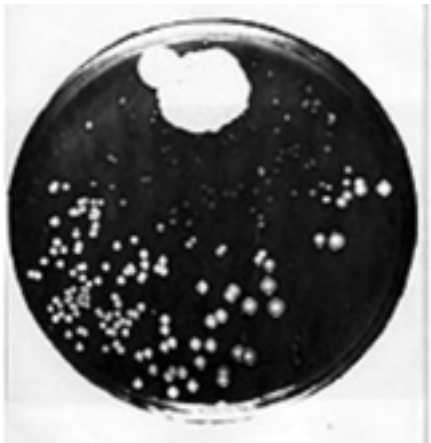
The *mecA* operon

The **repressor** MecI prevents transcription of the *mecA* gene and of *mecR1-mecI* in absence of an **inducer**. In presence of an inducer the transmembrane sensor-transducer MecR1 inactivates the repressor MecI and allows so transcription of *mecA* and thus PBP2a production.

Methicillin–resistance in staphylococci containing the *mecI-mecR1-mecA* **operon** is strongly repressed. Induction of PBP2a by methicillin or oxacillin is very slow. Such strains may appear to be susceptible, although they carry the methicillin resistance determinant. Cefoxitin is a better inducer. In the experiment we will demonstrate that PBP2a is produced after induction.

Background on penicillin action

Steps involved in bacterial **cell wall synthesis** are suitable targets for antibiotics. **Penicillin** inhibits cell wall biosynthesis and leads to lysis and death. Penicillin acts by preventing the **cross-linking of the peptide chains** of the bacterial **peptidoglycan**. The enzymes involved in these last steps of cell wall synthesis are called **penicillin-binding proteins (PBP).**



Growth inhibition and lysis of *Staphylococcus aureus* around a culture of *Penicillium notatum*, from http://www.pbs.org/wgbh/aso/databank/entries/dm28pe.html

Criteria for antibiotic resistance in bacteria:

Clinically	therapy resistance
Microbiologically	minimal inhibitory concentration (MIC)
Mutation	difference in resistance between parent and mutant
Genetically	acquired resistance determinant
Biochemically	resistance mechanism
Inherent	genus- or species-specific resistance

Resistance in bacteria can be determined:

- Phenotypically, by measuring the **minimal inhibitory concentration** (**MIC**) of the antibiotic. By comparing resistance between parent and mutant.
- Genetically, by showing the presence of a **resistance determinant sequence** or a particular mutation.
- Biochemically, by demonstrating the presence of the resistance determinant, or of the reaction leading to resistance.

In this experiment we will compare the production of PBP2a in induced and in non-induced cells of a methicillin-resistant *Staphylococcus aureus*.

Literature

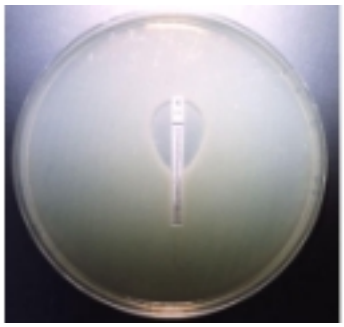
Archer G.: *Staphylococcus aureus*: a well-armed pathogen. Clin Infect Dis. 1998, 26 : 1179-81. Review.

Chambers HF.: Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev. 1997, 10: 781-91. Review.

www. Links

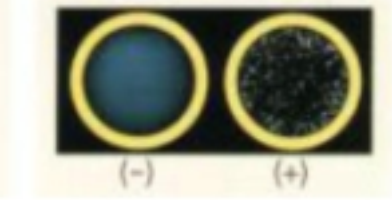
Practical work

The minimal inhibitory concentration of oxacillin will be determined by the **Etest method.**



Mueller-Hinton plate inocculated with a strain to be tested. The Estrip contains a gradient of an antibiotic. The minimal inhibitory concentration of the antibiotic can be read at the intersection of growth with the Estrip.

PBP2a will be determined by agglutination with latex beads coated with anti-PBP2a antibodies.



Latex agglutination: negative reaction left, positive reaction right.

Material and Experimental Protocols

The student will characterize a clinical MRSA isolate. As control you will use a susceptible *S. aureus* strain and an isogenic methicillin–resistant strain.
You will determine:

- the resistance of the strains to different antibiotics
- the MIC of oxacillin
- the presence or absence of PBP2a

The strains to be tested are streaked out on sheep blood agar. By holding the plates against the light you can see if they produce hemolysins. (Seen as a clear zone around the colonies).

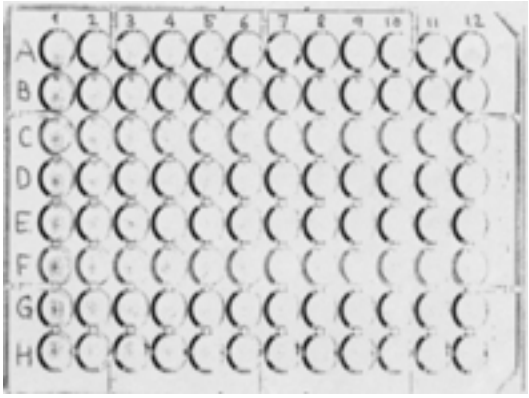
1. Preparing a working dilution of the strains
Pick up 3 to 5 colonies with a cotton tip applicator and suspend them into a tube containing sterile saline. Adjust the turbidity of the suspension to 0.5 McFarland (by comparing the turbidity to a reference tube containing the 0.5 McFarland standard). This working dilution contains approximately 10^8 bacteria/ml. Keep this solution for the following three experiments

1.1. Resistance tests
Dip a fresh cotton applicator in the working solution and swab two Müller-Hinton plates evenly to cover the entire surface. Change the angle by 120 ° and repeat this two times. The surface of the agar plates is now inoculated with a more or less homogenous layer of bacteria.
Applicate an oxacillin Estrip in the middle of one plate with a sterile forceps.
On the other plate stamp with an applicator paper disks impregnated with different antibiotics.
The plates will be incubated overnight at 37 °C and the inhibition zones around the antibiotics will be monitored in the next course:

- Measure the inhibition zones around the paper disks and determine if the strains are resistant or susceptible against the antibiotic.
- Determine the minimal inhibitory concentration of oxacillin that can be read at the intersection of the Estrip. Inspect the inhibition zone carefully for colonies appearing in the inhibition zone.

1.2. Induction of PBP2a
With the cotton tip applicator inoculate half of a sheep blood agar plate with the clinical isolate. Fractionate the bacteria with a plastic loop over the remaining half plate as will be demonstrated. Add a cefoxitin paper disk on the first fraction (cefoxitin is a penicillin-derivative, that can act as an inducer of *mecA*). This plate will be used in the next course to determine PBP2a by agglutination

1.3. MIC determination by microbroth dilution
The microbroth dilution allows determining MIC of antibiotics in broth. The microtiter plate contains a series of 12 wells in a row. Use one row for each strain.



1. Pipet 50 ml of broth into the wells 2 to 12 of the microtiter plate.
2. Add 50 ml of the antibiotic stock solution to the wells 1 and 2.
3. Make a geometric dilution of the antibiotic with the factor two: For this purpose: take 50 ml from well 2 and add it to well 3, mix by pipetting up and down. Continue this procedure up to well 10. Well 10 contains now 100 ml, remove and discard 50 ml from it.
Well 11 is the growth control with no antibiotic. Well 12 is the control for the sterility of the broth.

4. Dilute the working suspension of the clinical isolate 100 fold by adding 20 ml of the working dilution into 2 ml of broth.
5. Add 50 ml of the diluted working suspension into the wells 1 to 11. Cover the plate with a sticking plastic sheet and incubate over night at 37 °C. In the next course, the growth in each well can be read. The MIC of oxacillin corresponds to the lowest concentration of the antibiotic where no growth occurs.

2. PBP2a Agglutination

You will make agglutinations with latex beads loaded with anti-PBP2' antibodies to demonstrate the presence of PBP2a in the clinical *S. aureus* isolate. To be certain that PBP2a is expressed, the clinical MRSA isolate to be tested has been induced by cefoxitin.
To see the difference between induced and non-induced culture you will perform two agglutinations.
For probe **A** you take bacteria growing far away from the disk (uninduced culture).
For probe **B** we take the bacteria that grow close to the inhibition zone (induced culture).

Two extractions and agglutinations will be made, one with probe A and one with probe B:

2.1. Extraction of PBP2a

For each culture probe:

- Add 4 drops of **Extraction-Reagent 1** in a 1.5 ml Eppendorf tube
- Suspend a plastic loop full of bacteria in this tube
- Heat the suspension for 3 min to 95 °C and let it cool again to room temperature
- Add 1 drop of **Extraction-Reagent 2**, mix well
- Centrifuge for 5 min at 4500 rpm in an Eppendorf centrifuge

2.2. Latex agglutination

- For each probe prepare a **test card** with a ring for the probe and one ring for the control.
- Add in the first ring one drop of **Sensitized-Latex** and 50 ml of the supernatant from 2.1. above; mix with the plastic stick.
- Add one drop of **Control-Latex** in the second ring and 50 ml of the supernatant from 2.1.; mix with the plastic stick.
- Rotate the test-card for 3 min by hand and observe if you see agglutination.

2.3. Interpretation

- No agglutination with Sensitized-Latex and Control-Latex: PBP2a negative.
- Agglutination with Sensitized-Latex, no agglutination with Control Latex: PBP2 a positive.
- Agglutination with Control-Latex: undefined, repeat reaction.

Laboratory Rules & Precautions	*Staphylococcus aureus* is a normal inhabitant of skin and mucous membranes in 10 to 30 % of healthy people. It has, though, a pathogenic potential and can produce toxins and virulence factors. Care must be taken not to contaminate the environment. Aerosol formation has to be prevented. All waste must be sterilized before disposal. Hands must be washed and disinfected and surfaces wiped with 70 % ethanol after completion of the experiments.
Experiences gained	• To learn different methods of antibiotic resistance determination. • To see the difference between induced and uninduced cultures.
Timing	1st day 1.5 h, 2nd day 1 h
Reporting	• Report the inhibition zones and oxacillin MICs by Etest and by microbroth dilution of the different strains in a table. • Report if you see colonies in the inhibition zone. • Write down the observations of the agglutination experiment, and explain in a short sentence the results.
Questions to be answered	• How can methicillin resistant bacteria become constitutive producers of PBP2a? • Does penicillin act on resting cells? • Compare the advantages and disadvantages of genetic versus phenotypic determination of the resistance.