### TASK 4A: Microbial colonies on MSA plates (from TASK 3E)

You will analyse the growth and colony morphology of the unknown pathogen on MSA agar (from the plates you inoculated in the previous laboratory session, TASK 3E). You have also been provided with cultures of type strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* grown on MSA agar as controls. Record your observations in Table 15. (If necessary, refer back to Figure 2 for bacterial colony morphology descriptors.)

Table 15. Phenotypes of staphylococci grown on MSA plates

|  |  |  |
| --- | --- | --- |
| Isolate | Growth on MSA agar? | Characteristics of colonies |
| *Staphylococcus aureus*  (control) |  |  |
| *Staphylococcus epidermidis* (control) |  |  |
| Your unknown culture  (# \_\_\_\_\_\_) |  |  |

### Discussion questions: MSA results

What can you conclude about your unknown?

What further tests might you do to characterize and/or identify it?

### TASK 4B: Assaying for coagulase activity

Coagulase is an enzyme that has prothrombin-like activity and is capable of converting plasma fibrinogen to fibrin with resultant clot formation. Coagulase can be present in two forms, “free” and “bound” each having different properties and requiring different testing procedures. In this laboratory we will use the more rapid test for bound coagulase, the so-called slide test. As not all strains of *Staphylococcus aureus* express bound coagulase a negative test result requires the free coagulase (tube) test to be performed for absolute confirmation.

#### Protocol 4.1: Coagulase slide test.

1. Place a drop of sterile water on a glass slide.
2. Using a flamed loop, that has been allowed to cool, remove an isolated colony from the culture plate and gently emulsify on the slide with the drop of sterile water.
3. Place a drop of plasma immediately adjacent to the bacterial suspension and mix the two samples together.
4. Gently tilt the slide back and forwards, observing for the immediate formation of a white precipitate.

*Interpretation*

A positive reaction (formation of white precipitate) is typically detected 15 – 20 seconds. The test is considered negative if clumping is not observed within 2 – 3 minutes.

Record your results in Table 16.

Table 16. Coagulase activity of Staphylococcus sp.

|  |  |
| --- | --- |
| Microorganism | Coagulase test result –  carefully record what you observe |
| Positive control  (*Staphylococcus aureus*) |  |
| Negative control  (*Staphylococcus epidermidis*) |  |
| Your unknown |  |

Table 17. Characterization of your unknown organism

|  |  |  |
| --- | --- | --- |
| Biochemical Test | Observation | Positive or Negative? |
| Mannitol fermentation |  |  |
| Lysostaphin sensitivity |  |  |
| Coagulase activity |  |  |

### Discussion questions: identification of staphylococci

What can you conclude about your unknown organism?

### TASK 4C: MLVA-typing *Staphylococcus aureus*

If the previous tests have allowed you to determine that your unknown is *Staphylococcus aureus*, you will now be able to determine its identity more preciselyusing a technique called MLVA (multiple locus variable number of tandem repeats analysis), which can discriminate between different strains of *Staphylococcus aureus* based on different numbers of variable tandem repeats in specific loci. You will amplify these loci using PCR, and determine the size of the PCR products using agarose gel electrophoresis. The size of the locus (PCR product) reflects the number of repeats present in your strain. If more repeats are present, the PCR product will be larger (Figure 9). If your unknown was not *Staphylococcus aureus*, you will still be able to perform the MLVA analysis – use the *Staphylococcus aureus* culture that was provided as a positive control for the previous experiments.

You will be analysing five different loci in total; one in a single PCR reaction (the *spa* gene), and four in a multiplexed PCR reaction (the *clfA, cflB, sdr,* and *ssp* genes) as detailed in the following protocols.

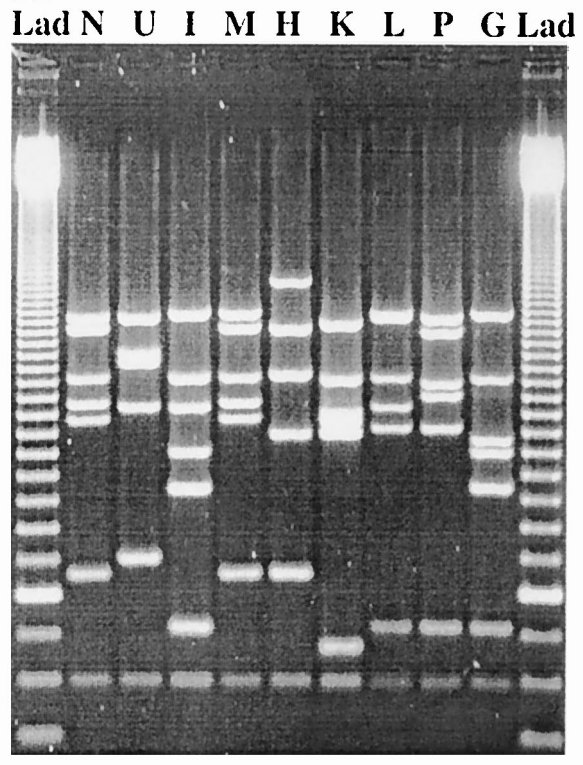


Figure 11. MLVA patterns of isolates 18 to 34. [taken from Sabat et al. (2003). J Clin Microbiol. 41: 1801 – 1804].

Table 18. Primers used in this experiment. (Sabat A et al., J Clin Microbiol. 2003 41: 1801). \* Product size is estimated with reference genome N315. Other strains are expected to have different sizes.

|  |  |  |  |
| --- | --- | --- | --- |
| Gene/locus | Primer name | Primer sequence (5’-3’) | Product size\* |
| *clfA* | clfA-F | GATTCTGACCCAGGTTCAGA | 1183bp |
| clfA-R | CTGTATCTGGTAATGGTTCTTT |
| *clfB* | clfB-F | ATGGTGATTCAGCAGTAAATCC | 828bp |
| clfB-R | CATTATTTGGTGGTGTAACTCTT |
| *sdr* | sdr-F | GTAACAATTACGGATCATGATG | 670bp |
| sdr-R | TACCTGTTTCTGGTAATGCTTT |
| *spa* | spa-F | AGCACCAAAAGAGGAAGACAA | 284bp |
| spa-R | GTTTAACGACATGTACTCCGT |
| *ssp* | ssp-F | ATCMATTTYGCMAAYGATGACCA | 173bp |
| ssp-R | TTGTCTGAATTATTGTTATCGCC |

***Setting up PCR reaction mixtures***

* Work on ice.
* Pipet carefully and accurately.
* Watch what you touch – you are covered with nucleases and your own *Staphylococcus aureus*!
* Stay organized – keep track of what you have added to your reaction and what still needs to be added.
* Label your tubes – there will be 96 on the thermocycler altogether!

#### Protocol 4.2: Set up single PCR (spa gene).

The *spa* gene must be amplified separately as these primers do not perform well in the multiplex PCR reaction. The single PCR mixture contains: primers for the gene to be amplified (the *spa* gene), the template DNA, the polymerase enzyme (*Taq*), dNTPs, the reaction buffer, and PCR-grade water.

Table 19. Reaction mixture for the spa single PCR

|  |  |
| --- | --- |
| Component | Volume for 1 reaction |
| 5X Taq buffer | 4 μl |
| PCR grade water | 13.75 μl |
| F primer | 1 μl |
| R primer | 1 μl |
| *Taq* polymerase | 0.25 μl |
| Total volume | 20 μl |

1. Label your PCR tube carefully with the gene name (*spa*) and your bench number.
2. Use a white thin (10 μl) tip to pick one big isolated colony from the plate provided to you, and place this at the bottom of your PCR tube. Add 20 μl PCR master mix.
3. Keep the reaction mixture on ice until the whole class is ready to proceed to the amplification step (thermocycler).

#### Protocol 4.3: Set up multiplex PCR (clfA, clfB, sdr and ssp genes).

The multiplex PCR mixture contains: primers for each of the genes to be amplified (the *cflA, cflB, sdr,* and *ssp* genes), the template DNA, the polymerase enzyme (*Taq* polymerase), dNTPs, and the reaction buffer.

Table 20. Reaction mixture for the multiplex PCR

|  |  |
| --- | --- |
| Component | Volume for 1 reaction |
| PCR grade water | 7.75 μl |
| 5X Taq buffer | 4 μl |
| cflA-F primer | 1 μl |
| cflA-R primer | 1 μl |
| cflB-F primer | 1 μl |
| cflB-R primer | 1 μl |
| sdr-F primer | 1 μl |
| sdr-R primer | 1 μl |
| ssp-F primer | 1 μl |
| ssp-R primer | 1 μl |
| *Taq* polymerase | 0.25 μl |
| Total volume | 20 μl |

1. Label your PCR tube carefully, including your bench number.
2. Use a white thin (10 μl) tip to pick one big isolated colony from the plate provided to you, and place this at the bottom of your PCR tube. Add 20 μl PCR master mix.
3. Keep the reaction mixture on ice until the whole class is ready to proceed to the amplification step.

***PCR amplification for both reactions***

Amplification of DNA fragments will be performed with an initial denaturation at 94°C for 10 minutes followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and one minute at 72°C, with a final extension at 72°C for 5 minutes on a Bio-Rad DNA-Engine thermocycler. This PCR programme takes about 2½ hours to run.

#### Protocol 4.4: Make an agarose gel.

1. Prepare a gel tray. Use tape to seal off the top and bottom ends. Insert the comb in the slots in the tray. Check with a demonstrator before proceeding.
2. A 2% agarose solution has been made for you as follows: 2 g of agarose were added to a glass flask, and 100 ml 1X TAE buffer. The agarose solution was heated in a microwave till agarose is completely dissolved in the buffer, and then cooled down to about 50°C.
3. Pour the agarose into your prepared gel tray and leave it to set.

*Gel electrophoresis*

After the thermocycler run has finished, your samples will be run on your gel for you as follows: we will add 5 µl of *spa* PCR product to the multiplex PCR products and then run 16 µl of the combined reaction on the 2% agarose gel. Bioline 100bp DNA ladder (BIO-33056) will be used for each of the gels. We will then take gel images with the “gel doc system” and post your data to the BM327 microbiology class sub-page.

### Discussion questions: MLVA-typing

### TASK 4D: Observing and recording results (from TASK 3A)

You will need to observe the growth of your unknown and control strains on selective/differential media and record your results in Table 21.

Table 21. Visual observation of microbial colonies incubated on MacConkey agar

|  |  |
| --- | --- |
| Microorganism cultured | Visual observations (colony characteristics) |
| *Escherichia coli* |  |
| *Salmonella enteritidis* |  |
| *Your unknown* |  |

### Discussion questions: MacConkey results

What can you conclude about your unknown based on this result?

### TASK 4E. Observing and recording results (from TASK 3C)

You will also need to observe the growth of your unknown and control strains in aerobic and anaerobic conditions and record your results in Table 22 and Table 23.

Table 22. Visual observation of microbial growth in fluid thioglycollate medium.

|  |  |  |
| --- | --- | --- |
| Microorganism Cultured | Visual observations (turbidity/growth, colour) | Conclusions |
| *Your unknown* |  |  |
| *Escherichia coli* |  |  |
| *Clostridium* |  |  |

Table 23. Visual observation of microbial growth on stiff blood agar plates incubated aerobically and anaerobically

|  |  |  |
| --- | --- | --- |
| Microorganism cultured | Visual observations (colony characteristics) – plates cultured aerobically | Visual observations (colony characteristics) – plates cultured anaerobically |
| *Your unknown* |  |  |
| *Clostridium* |  |  |

### Discussion questions: anaerobic growth results

What can you conclude about your unknown based on this result?

### TASK 4F: Analysing antibiotic resistance of *P. aeruginosa* isolates

Bacteria in biofilms tend to be more resistant to killing and/or growth inhibition by antimicrobial agents. A number of different mechanisms have been proposed to explain this increased resistance, including that the biofilms may provide a measure of physical protection, i.e. with an extracellular matrix shielding the cells from contact with the antimicrobial agent. Alternatively, the cells present in a biofilm may have altered their gene expression/physiological state such that they are now more resistant to the antimicrobial. In this lab, you will test your *Pseudomonas aeruginosa* isolates to determine whether they are sensitive or resistant to the aminoglycoside antibiotic gentamicin.

#### Protocol 4.5: Determining the minimal inhibitory concentration of an antibiotic

1. Prepare a seed lawn for each of your three *Pseudomonas aeruginosa* isolate as follows: pipette 100 µl of the supplied culture onto the centre of a Mueller Hinton agar plate and use a sterile plastic spreader to distribute the culture evenly over the surface of the agar. The liquid should be completely absorbed into the agar before you add the antibiotic disks in step 5.
2. Obtain an empty petri dish containing fifteen sterile filter discs.
3. In four sterile 1.5 ml tubes, prepare dilutions of the supplied solution of gentamicin (1 mg/ml). You will be adding 20 µl of each dilution to a filter disk, but it is always a good idea to prepare a slightly larger volume to account for any pipetting errors - e.g. prepare 25 µl for each filter disk. Prepare the dilutions so that you will end up with 4 µg, 8 µg, 12 µg and 16 µg of gentamicin when you add 20 µl of the dilution to a filter disk.

Equation: C1 x V1 = C2 x V2 [C, concentration; V, volume]

Example: 4 µg/20 µl x 25 µl = 1 mg/ml x ???

Using this equation, complete Table 24 and then prepare the dilutions:

Table 24. Calculation of antibiotic dilutions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Final amount on disc | 4 µg | 8 µg | 12 µg | 16 µg |
| Volume stock solution |  |  |  |  |
| Volume H20 |  |  |  |  |

### Discussion questions: MIC determination

### Competency check: streak plate.

One of the lecturers will assess your streak plate(s) for competency according to the following criteria:

Streak plate competency check 3:

* Well-streaked plate, with several well-separated and distinct individual colonies
* Streaking technique needs improvement: does not make good use of the space available on the plate
* Streaking technique needs improvement: no/few single colonies obtained
* Streaking technique needs improvement: probable faults in aseptic technique
* Streaking technique needs improvement: other \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Labelling technique:

* Clear, legible, complete label
* Labelling is illegible/unclear
* Label placed incorrectly: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
* Label is missing key information: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

You should use the feedback provided by the lecturer on your streak plate to improve your technique. You will be given several more opportunities to pass the streak plate competency test over the course of this module.