17th INTERNATIONAL BIOLOGY OLYMPIAD
9 - 16 JULY 2006
Río Cuarto – República Argentina

PRACTICAL TEST

4

MICROBIOLOGY

Student Code:	

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General remarks about the practical tests

DEAR PRATICIPANS

The practical test are organized in four different laboratories.

Nº 1- Plant Anatomy, Systematics and Physiology

Nº 2- Animal Anatomy, Ecology and Systematics

Nº 3- Biocheminstry

Nº 4- Microbiology

- You have **1 hour** in laboratories Nº 1 and Nº 2.
- You have **1 hour 30 minutes** in laboratories Nº 3 and Nº 4.
- You can score maximum **40 points** in each laboratories, which means a total of 160 points for the practical test.

Good luck !!!!!!!

Practical test

MICROBIOLOGY

There are different systems of bacterium classification, but the most commonly used is the published in Bergey's Manual of Determinative Bacteriology.

A working outline for the identification of a bacterial strain from the biochemical point of view is proposed below:

1) Isolate the strain and obtain a pure culture.

2) Carry out a microscopic examination of living cells and also of Gram stained smears. The morphology and type of Gram staining of the microorganism under study is thus determined. It is also important to determine the presence of clusters, spores and any other morphological characteristics that may be of interest.

3) Determine the nutritional characteristics (in general they come off from the methods used in the previous isolate and culture): photoautotrophs, photoheterotrophs, chemiautotrophs, chemiheterotrophs.

4) Conduce primary tests: The following group of tests, called primary tests, are used to determine the genus, group of genera or in some cases, the family to which an isolate belongs to. The primary tests are, beside Gram staining and morphology observation, the determination of catalase, oxidase, glucose fermentation, and motility, among others.

Reagents and Equipment

1. Dropping bottle with Gentian Violet (ready to use)
2. Dropping bottle with Lugol (ready to use)
3. Dropping bottle with Gram decolorizer (ready to use)
4. Dropping bottle with Safranin (ready to use)
5. Dropping bottle with Distilled water
6. 1 tube rack
7. 2 Kahn tubes containing a culture grown in Luria-Bertani medium of organisms A and B.
8. 2 Lab gloves

9. Respiratory mask
10. Marker pen
11. Paper napkin
12. 1 Bunsen burner
13. Microscope
14. Loop
15. 4 Slides
16. Tray with slide holder
17. 1 plastic bottle with water for rinsing
18. 1 disposable glass
19. 1 dropping bottle with immersion oil
20. 1 dropping bottle with 3% H_2O_2
21. 2 Luria-Bertani agar plates inoculated with organisms A and B.
22. 1 Eppendorf tube with 2 oxidase disks
23. 1 pair of tweezers
24. 2 Kahn tubes
25. 1 Kahn tube with a stopper containing sterile distilled water.
26. 1 plastic Pasteur pipette.
27. 3 plates with eosin methylene blue agar (EMB) medium (one of them inoculated with organism A, another inoculated with organism B and the last one without inoculation)
28. 3 tubes with phenylalanine (one of them inoculated with organism A, another inoculated with organism B and the last one without inoculation).
29. 1 Dropper containing 10% ferric chloride
30. 3 Kahn tubes with SIM (hydrogen Sulfide Indole Motility) medium (one of them inoculated with organism A, another inoculated with organism B and the last one without inoculation)
31. 1 Dropping bottle containing Indole reagent
32. 3 Kahn tubes containing UREA broth (one of them inoculated with organism A, another inoculated with organism B and the last one without inoculation)
33. 3 Kahn tubes with motility indole ornitine medium (MIO) (one of them inoculated with organism A, another inoculated with organism B and the last one without inoculation)

34. Clock located in view of all the students in the laboratory.

Caution:

You must be careful in the manipulation of media and reagents since the quantities provided allow performing this practical test only once.

If you work carelessly, with abrupt movements, far from the burner, you will contaminate the medium thereby preventing you from obtaining good results.

You will perform the biochemical tests which basis and interpretation are detailed below by using the media, reagents, and the given bacteriological information (charts and diagrams)

Note: Do not discard the tubes with organisms A and B. You will use them in task 2.

TASK 1: Perform Gram-staining in organisms A and B.

EXPERIMENTAL PROCEDURE

Introduction:

Gram stain differentiates between two major bacterial cell wall types. Some bacterial species, because of the chemical nature of their cell walls, have the ability to retain the crystal violet even after the treatment with an organic decolorizer such as a mixture of acetone and alcohol.

Gram stain technique

1. Make a thin smear of the material to study and allow to air dry.

2. Fix the material to the slide so that it does not wash off during the staining procedure by passing the slide three or four times through the flame of a Bunsen burner.

3. Place the smear on a staining rack and overlay the surface with Gentian Violet solution.

4. After 30 seconds of exposure to the Gentian Violet solution, wash thoroughly with running water.

5. Next, overlay the smear with Gram's iodine solution (lugol) for 30 seconds.

6. Hold the smear between the thumb and forefinger and flood the surface with a few drops of the acetone alcohol decolorizer until no violet color washes off. This usually requires 10 seconds or less time.

7. Wash with running water and again place the smear on the staining rack. Overlay the surface with safranin counterstain for 20 seconds. Wash with running water.

8. Place the smear in an upright position in a staining rack, allowing the excess water to drain off and the smear to dry.

9. Examine the stained smear under the 100 x (oil) immersion objective of the microscope.

10. When you focus the microscope call the assistant.

Result

SELECT THE CORRECT ANSWER, FILLING THE CORRESPONDING BOX

Organism	Gram staining		Assistant revision
A	Positive	Negative	
B	Positive	Negative	

Question

Gram-variability

A) is a term which can be used where two Gram reactions are seen due to an error in the staining procedure.

B) applies to an organism which changes its cell wall structure from the Gram-positive type to the Gram-negative type as the culture ages.

C) applies to what is ultimately seen when cells in a culture of gram-positive bacteria lose the ability to retain the primary stain during the decolorization process.

D) indicates a mixed (i.e., impure) culture.

Write the letter corresponding to the correct answer on the dotted line below:

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TASK 2: Biochemical characterization of organisms A and B.

In this part of the practical work you will determine, by means of metabolic tests (already provided or performed by you), the family and genus of the two organisms labeled as A and B.

Catalase Reaction:

Some bacteria contain flavoproteins that reduce the oxygen resulting in the production of hydrogen peroxide (H_2O_2) or superoxide (O_2 -), which are extremely toxic since they are powerful oxidizers able to destroy the cellular constituents in a short time. Many bacteria possess enzymes that offer protection against these toxic compounds.

Technique

Perform the catalase test to organisms A and B by adding two drops of H_2O_2 to a bacterial suspension (3 loopfuls of the liquid culture labeled as LB-A and LB-B) placed on the slides.

Note: Write the obtained results in the Biochemical test table using + (for a positive reaction) or – (for a negative reaction)

Question

Which of the following reactions is carried out by the catalase enzyme?

1) $H_2O_2 + NADH + H^+ \rightarrow 2\ H_2O + NAD^+$

2) $H_2O + H_2O \rightarrow 2H_2O + O_2$

3) $O_2^- + O_2^- + 2H+ \rightarrow H_2O_2 + O_2$

4) $4\ O_2^- + 4H^+ \rightarrow 2H_2O + 3\ O_2$

Write the number corresponding to the correct answer on the dotted line below:

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Oxidase Reaction:

Test used for the detection of the cytochrome-c-oxidase enzyme, which is present in different genera, e.g. *Pseudomonas* spp., *Neisseria* spp., *Moraxella* spp., *Vibrio* spp., *Aeromonas* spp.

Oxidase discs contain dimethyl-para-phenylene-diamine, which is the substrate of cytochrome-c-oxidase enzyme.

Organisms possessing this enzyme, in the presence of atmospheric oxygen and the substrate contained in the oxidase discs give a red-fuchsia color.

Technique

Perform the oxidase test to organism A and B according to the following instructions:

Oxidase test will be carried out using tubes. From a pure culture, prepare a heavy suspension in 0.2 ml of sterile distilled water, and add one oxidase disc.

Note: Prepare the bacterial suspension starting from 3 colonies of each one of the plates labeled as LB-A and LB-B respectively.

Results

Generally, within the first minute and at room temperature, positive results are detected. A delayed reaction, evidenced after 2 minutes must be considered a negative result.

Positive: discs show a red-fuchsia color.

Negative: no changes in disc color.

Note: Write the obtained results in the Biochemical test table using + (for a positive reaction) or – (for a negative reaction)

EOSIN METHYLENE BLUE (EMB) AGAR

This medium, is used for the selective isolation of fast growing and with scarcely nutritional requirements Gram-negative bacteria.

It allows the growth of all Enterobacteriaceae members.

Purpose

This medium combines the Holt-Harris formulation with the Levine´s one to improve the selective isolation of Enterobacteriaceae and other Gram-negative bacterium species. The differentiation between lactose and/or sucrose fermenter organisms from those organisms which do not ferment them is possible due to the presence of the indicators eosin and methylene blue. Also, these indicators inhibit the growth of several Gram-positive bacteria.

Many strains of *Escherichia coli* and *Citrobacter spp.* show colonies with a greenish metallic sheen in this medium.

Lactose and/or sucrose fermenter organisms show colonies with a dark center surrounded by a blue or pink color, while lactose and/or sucrose non fermenter

organisms show colorless colonies.

This medium also allows the growth of different organisms in addition to the growth of the Enterobacteriaceae members, and may be generally differentiated by the appearance of their colonies.

Instructions

Using the EMB plates provided (labeled as EMB-A and EMB-B for organisms A and B respectively), determine the sucrose and/or lactose utilization for organisms A and B.

Note: Write the obtained results in the Biochemical test table using + (for a positive reaction) or – (for a negative reaction).

Question

Fermentation

A) results in production of acid and possibly gas from the breakdown of sugars.

B) is associated with the type of growth of facultative anaerobes in Thioglycollate Medium where growth is less dense in the anaerobic region.

C) is generally associated with a positive catalase reaction for an organism.

Write the letter/s corresponding to the correct/s answer/s on the dotted line below:

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Phenylalanine agar (Tubes labeled as Ph)

Phenylalanine agar is recommended for the detection of production of phenylpyruvic acid from phenylalanine by deamination. A positive reaction results in a green coloration after the application of 10% ferric chloride.

Instructions

Add 4 or 5 drops of the ferric chloride solution to the phenylalanine slants agar tubes (labeled as Ph-A and Ph-B for organisms A and B respectively). As the reagent is added rotate the tubes. An intense green color appearing within 10 minutes indicates the presence of phenylpyruvic acid.

Note: Write the obtained results in the Biochemical test table using + (for a positive reaction) or – (for a negative reaction).

Hydrogen Sulfide indole motility medium (SIM) (Tubes labeled as SIM A and SIM B)

This medium is used for the detection of hydrogen sulfide, indole production, and motility in the same tube. Hydrogen sulfide production in this medium is originated from thiosulphate or sulphate reductases and not by cystine desulfhidrases. Any blackening along the line of inoculation is considered as a positive hydrogen sulfide reaction, and it usually appears between 18-24 hours of inoculation. Motile cultures in SIM medium show diffuse growth away from the line of inoculation. This is an appropriated medium for the detection of Listeria's characteristic "umbrella-like" movement. The high content of trypteine in this medium makes it very suitable for detection of indole production.

Instructions

Using the SIM tubes provided (labeled as SIM-A and SIM-B for organisms A and B respectively), determine the production of hydrogen sulfide and indole, as well as the motility for organisms A and B.

For indole production detection, add 5 drops of the reagent (labeled as indole) to the heavy growth obtained in SIM tubes. A pink color promptly developed indicates the presence of indole.

Note: Write the obtained results in the Biochemical test table using + (for a positive reaction) or – (for a negative reaction).

Question

A negative result for motility

A) is indicated if growth occurs only along the line where the medium was stab-inoculated.

B) should be confirmed by a wet mount of a young culture of the same organism.

C) may exhibit growth over the surface of the medium.

D) may occur for strictly aerobic, motile organisms.

Write the letter/s corresponding to the correct/s answer/s on the dotted line below:

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UREA BROTH

This medium is suitable for the differentiation of urease producing organism.

Instructions

Using the urea broth tubes provided (labeled as UREA-A and UREA-B for organisms A and B respectively), determine the production of urease for organisms A and B.

Note: Write the obtained results in the Biochemical test table using + (for a positive reaction) or – (for a negative reaction).

SIMMONS CITRATE AGAR

It is a medium capable to differentiate between bacteria harboring citrate permease enzymes and those that do not harbor such enzymes.

Instructions

Using the SIMMONS CITRATE tubes provided (labeled as SC-A and SC-B for organisms A and B respectively), determine the presence of growth for organisms A and B.

Note: Write the obtained results in the Biochemical test table using + (presence of growth) or – (absence of growth).

Motility Indole Ornitine (MIO) Medium

The reactions in this medium are observed as follows:

- Ornithine Decarboxylation (ODC). Observe the lower three-quarters (anaerobic region) of the medium for change in color of the pH indicator; growth must be present in this part of the tube for correct analysis of result:
    - Gray, blue or purple color: Positive reaction for ornithine decarboxylation – formation of a highly alkaline product, over-neutralizing the acid produced from glucose fermentation.
    - Yellow color: Negative reaction. Yellow color is due to the "default" acid production from glucose fermentation.

Instructions

Using the MIO tubes provided (labeled as MIO-A and MIO-B for organisms A and B respectively), determine the production of ornithine decarboxylase enzyme for organisms A and B.

Note: Write the obtained results in the Biochemical test table using + (for a positive reaction) or – (for a negative reaction).

Results:

Write the results of the biochemical tests in the following table

Organism	catalase	lactose	sucrose	motility	indole	SH_2	Phenyl alanine	ODC	Ureasa	citrate	oxidase
A											
B											

Using the tables in the annex indicate

	Family	Genus
Organism A		
Organism B		

Questions

1. You have cultures of five organisms as listed below. However, the labels of the tubes have come off and you need to re-label the tubes correctly! First, you must consider the various reactions you know for the organisms in question:

genus	Gram stain	shape	catalase reaction	glucose fermentation	lactose fermentation	phenylalanine deaminase	citrate utilization
Bacillus	+	rod	+	+ or –	?	?	?
Staphylococcus	+	coccus	+	+	?	?	?
Enterobacter	–	rod	+	+	+	–	+
Morganella	–	rod	+	+	–	+	–
Pseudomonas	–	rod	+	–	–	–	?

a. The results obtained from what specific laboratory procedure will differentiate *Bacillus* and *Staphylococcus* from each other and also from the remaining three genera?

A) Glucose fermentation

B) Citrate utilization

C) Catalase reaction

D) Gram stain

Write the letter corresponding to the correct answer on the dotted line below:

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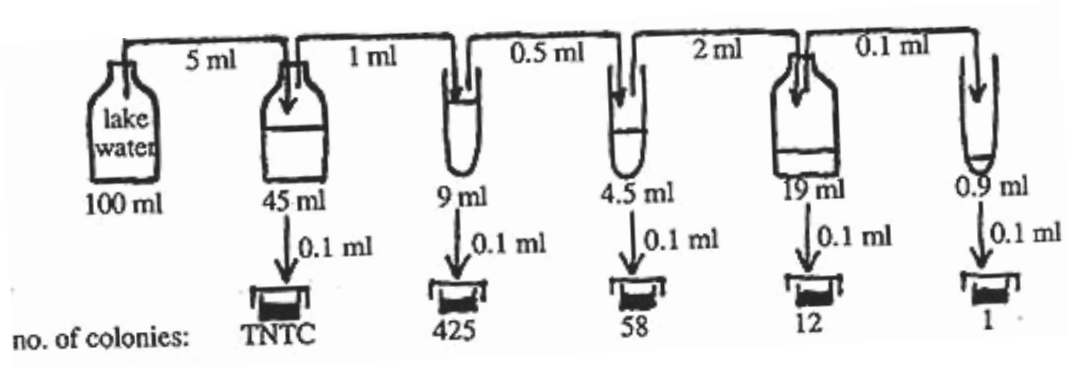
2. Consider the following dilution scheme:



a. Report the total number of CFUs (colony forming units) in the entire 100 ml amount of the original lake water sample. (TNTC=too numerous to count.)

A) $5.8\ 10^{7}$ cfu / 100 ml

B) $4.25\ 10^{7}$ cfu / 100 ml

C) $1.2\ 10^{8}$ cfu / 100 ml

Write the letter corresponding to the correct answer on the dotted line below:

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b. Would you expect any change in the answer of the above problem if the first dilution was made by adding one ml of sample to 9 ml of diluent?

A) Yes

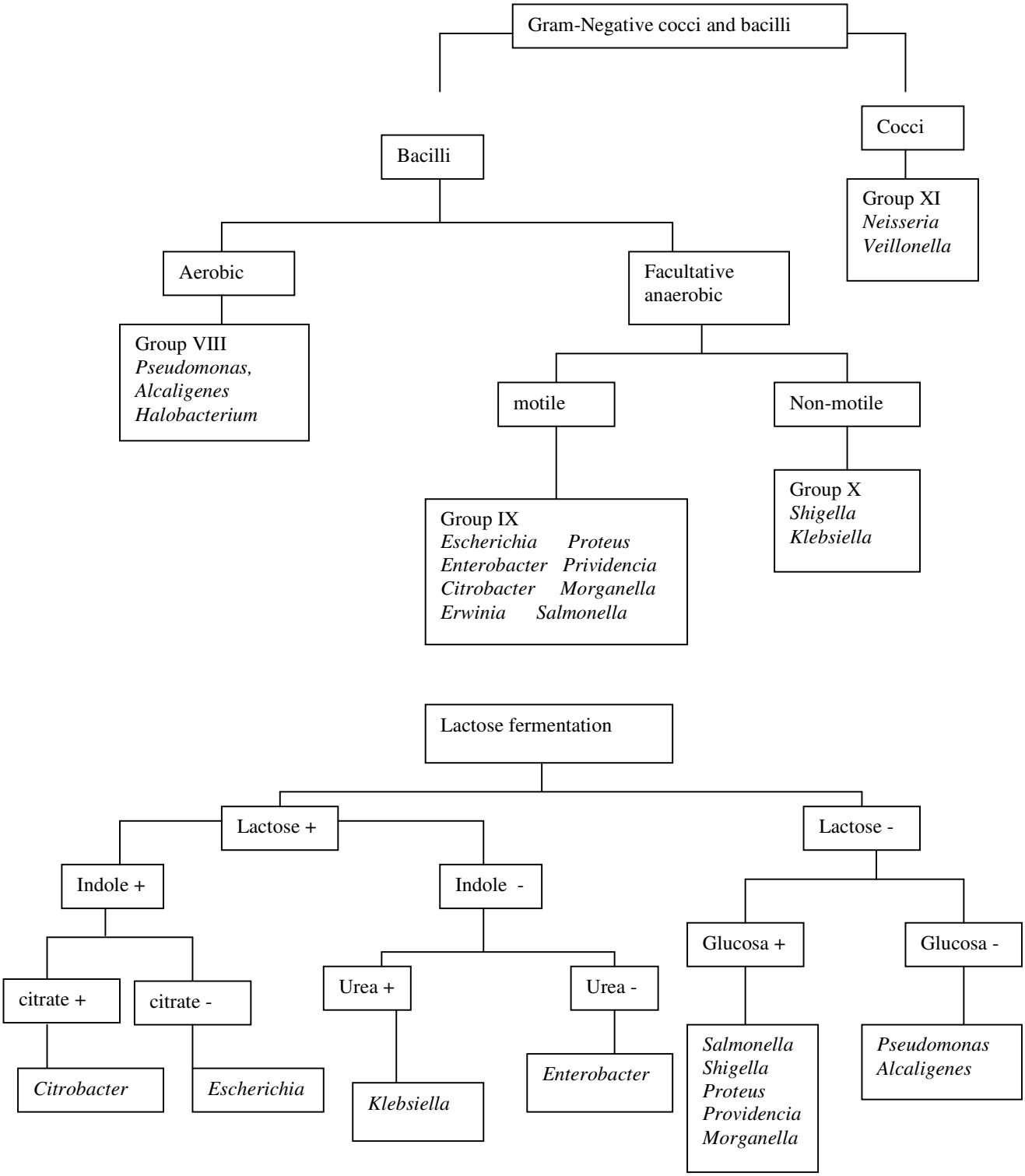
B) No

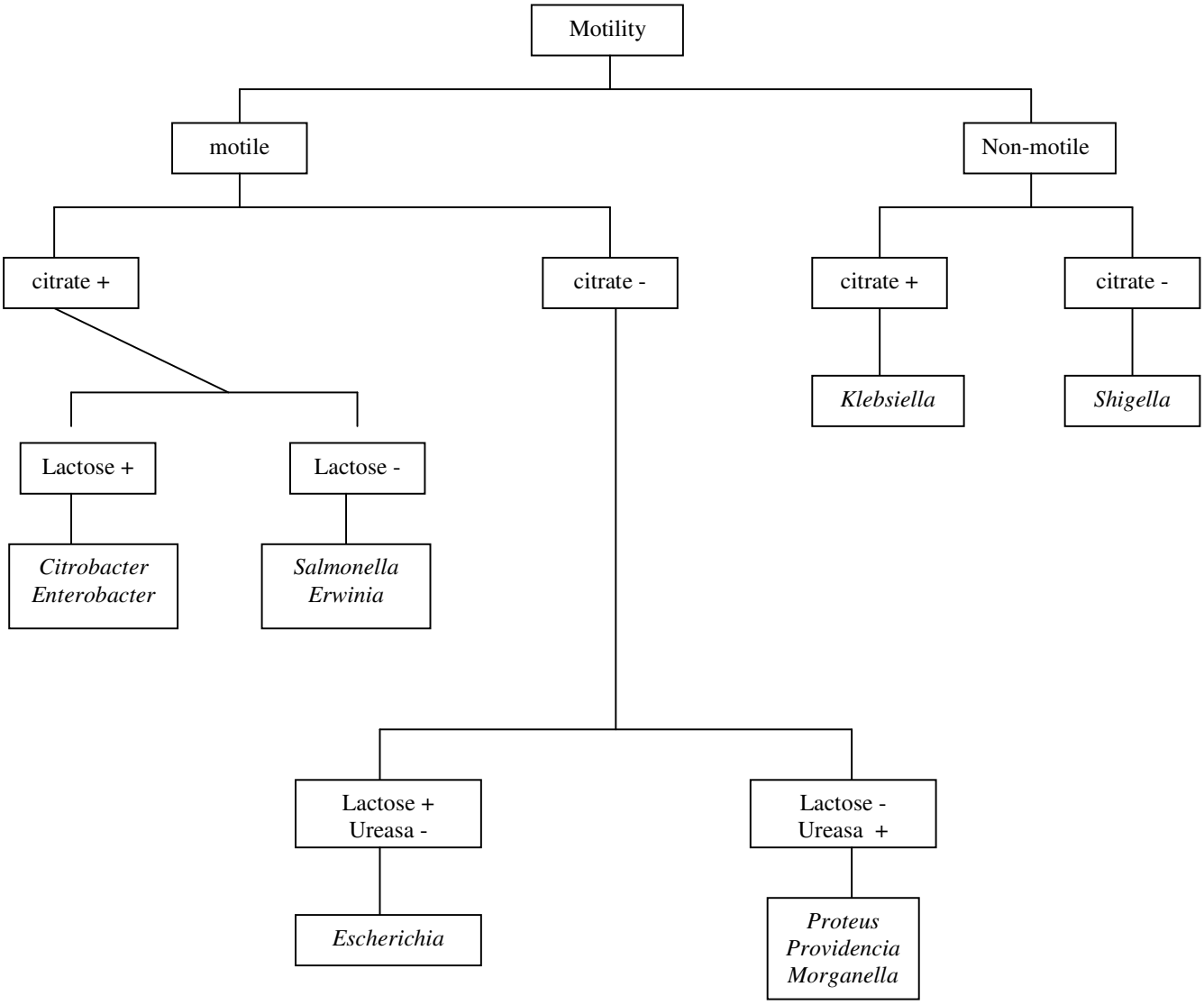
Write the letter corresponding to the correct answer on the dotted line below:

……

Annex 1

Gram stain (fresh culture)	+	+	+	+	+	+	+	+	–	–	–	–	–
shape	coccus	coccus	coccus	coccus	rod	rod	rod	rod	rod	rod	rod	rod	coccus
grouping	clusters	clusters	chains	Tetrads									pairs
aerobic growing	+	+	+	+	+	–	+	+	+	+	+	+	+
anaerobic growing	–	+	+	+	+	+	+	–	–	–	+	+	–
motility	–	–	–	–	–	+ or –	+ or –	+ or –	+ or–	+ or –	+ or -	+	–
catalase	+	+	–	–	–	–	+	+	+	+	+	+	+
oxidase									+	+	–	+	+
fermentation of glucose to acid or acid+gas	–	+	+	+	+	+ (or –)	+	–	–	–	+	+	–
Micrococcus	**X**												
Staphylococcus		**X**											
Streptococcus			**X**										
Lactococcus			**X**										
Enterococcus			**X**										
Clostridium						**X**							
Bacillus							**X**	**X**					
Alcaligenes									**X**				
Pseudomonas										**X**			
Enterobacterias											**X**		
Aeromonas												**X**	
Chromobacterium												**X**	
Neisseria													**X**





Family	Genus		oxidation									
		catalase	lactose	sucrose	motility	indole	SH_2	Phenyl alanine	ODC	Ureasa	citrate	oxidase
Escherichieae	*Escherichia*	+	+	+	+	+	-	-	-	-	-	-
	Shigella	+	-	-	-	+	-	-	-	-	-	-
Salmonellaeae	*Salmonella*	+	-	-	+	-	+	-	+	-	-	-
	Citrobacter	+	+ / -	-	+	+	+	-	+	-	+	-
Proteeae	*Proteus*	+	-	+	+	+ / -	+	+	+	+	+ / -	-
	Morganella	+	-	-	+	+	-	+	+	+	-	-
Klebsielleae	*Enterobacter*	+	+	+ / -	+	-	-	-	+	+ / -	+	-
	Serratia	+	+ / -	+ / -	+	-	-	-	+ / -	+	+	-
	Klebsiella	+	+	+	-	-	-	-	-	+	+ / -	-
Pseudomonaceae	*Pseudomonas*	+	?	-	+	-	-	+ / -	+ / -	+ / -	+ / -	+