# Exercise 7-A
# INTRODUCTION TO PROKARYOTES AND ENRICHMENTS FOR SELECTED BACTERIA FROM THE ENVIRONMENT

## Introduction

The prokaryotes, Bacteria and Archaea are ubiquitous organisms that as a group are the most ecologically and metabolically diverse life forms known. The term **prokaryote** (meaning before a nucleus) was introduced in 1937 to distinguish cells without nuclei from those with, but was not entirely correct. In 1961, **Roger Stainer** provided a more accurate definition for prokaryotes, i.e., cells that do not have a nuclear membrane surrounding their nucleoplasm, yet the name remains controversial. Some bacteriologists consider the name Prokaryote inappropriate because it is based on a characteristic these cells do not have, i.e. a true nucleus. Because individual prokaryotic cells are simple in form and often not morphologically distinctive, microbiologists developed a variety of methods for identifying them using features other than morphology. For prokaryotes easily grown in vitro, these methods included enzymatic testing, chromatography, serological typing, phage typing and other techniques involving laboratory experimentation/investigation. The ***Bergey's Manual of Determinative Bacteriology*** (first published in 1923) provided a variety of identification schemes and became an important reference. In the eighth edition of the *Bergey's Manual* (published in 1978) prokaryotic organisms were divided into two major categories, the bacteria and the cyanobacteria (archaea were not yet recognized as unique). This volume identified 1800 species in 245 genera, but categorized these into groups or parts more for convenience than as an indication of **phylogeny** (evolutionary history). This edition of the Bergey's manual also indicated that many prokaryotes remained unclassified.

A newer version of the *Bergey's Manual* (a four volume set completed in 1989) identified many more examples of prokaryotic organisms, and attempted to categorize these according to phylogenetic relationships. This internationally recognized manual contained names and descriptions of organisms, diagnostic keys, tables for identification, and a variety of other information. In these references, classification was based on numerous criteria including; cell wall composition, shape, size, and arrangement of cells, Gram stain, motility, mode of division, nutrition and metabolism, gas requirements, temperature requirements, environmental relationships, and biochemical analysis. Information about the isolation and maintenance of various cultures was also included. Because these volumes include much more than identification (determinative) schemes, they were called the ***Bergey's Manual of Systematic Bacteriology.*** This edition of the Bergey's manual was divided into four volumes for ease of handling, and grouped prokaryotes into categories as follows; the ordinary Gram-negative bacteria (volume 1), the ordinary Gram-positive bacteria (volume 2), bacteria with unusual properties (volume 3) and bacteria with filamentous morphology (volume 4). The Archae were included in volume three as Archaeobacteria (bacteria with unusual properties).

Recent technological advances in gene amplification, nucleotide sequencing and comparative data analysis have made it possible for microbiologists to begin unraveling the complex phylogeny of prokaryotic cells. Information obtained through the analysis of 16S and 18S ribosomal-DNA nucleotide sequences indicates that there are actually three major categories of cells, and many more species of prokaryotes than initially believed. Since all cells contain ribosomes, and all ribosomes are similar (both structurally and functionally), these particles provide an excellent source of material for determining the evolutionary history (phylogeny) of cells. The small ribosomal subunit (30S in prokaryotes and 40S in eukaryotes) contains a strand of RNA that is around 1600 or 1800 bp in length, and this is the subject of intensive investigation.

By analyzing and comparing the nucleotide sequences in these RNA molecules (or more often the DNA coding for these molecules), researchers have found that all cells can be divided into three distinctively different categories. Two of these categories are prokaryotic (the Bacteria and the Archaea) while the third includes all types of eukaryotic cells. Using this and other data, microbiologists have developed a new version of the Bergey's Manual, *Bergey's Manual of Systematic Bacteriology* 2nd Edition (a seven volume set when completed) containing the most complete version of prokaryotic taxonomy to date. Although this new Bergey's Manual includes a tremendous number of organisms, microbiologists believe that only about 1-10% of prokaryotic microbes have been identified and characterized.

Because a complete set of the new Bergey's manual (2nd Edition) is not yet available in this laboratory (not all volumes have been published), students will be using portions of both the first and second editions of the *Bergey's Manual of Systematic Bacteriology* as primary references. Within each volume of the first edition, the bacteria are divided into "**sections**" on the basis of common characteristics. The term "section" was used in the place of an established **taxonomic rank** (taxon) because the evolutionary relationships between and within sections were not thoroughly understood. Individual sections often include **taxa** such as families and orders, but the phylogenetic relationships between organisms within these categories are not necessarily valid. The organisms most commonly encountered in this laboratory occur in the volumes and groups listed below. The Part numbers in parentheses refer to the determinative manual, 8th edition.

Volume 1
The Gram-negative aerobic rods and cocci (Part 7 & Part 10)
The Gram-negative facultatively anaerobic rods (Part 8)

Volume 2
The Gram-positive cocci (Part 14)
The endospore forming rods and cocci (Part 15)
The Gram-positive regular non-spore forming rods (part 16)
The Gram-positive irregular rods (part 16)
The Mycobacteria (Part 16)

Volume 3
The anoxygenic photosynthetic bacteria (Part 1)
The oxygenic photosynthetic bacteria (Part 1)

Volume 4
The Actinomycetes and related organisms (Part 17)

Those volumes of the second edition currently available (Volumes One and Two) include the **Archaea**, **Deinococcus-Thermus**, **Cyanobacteria**, anoxygenic phototrophs (Volume One), and the **Proteobacteria** (Volume Two). Commonly encountered Gram-negative bacteria including both the aerobic and facultatively anaerobic genera described in Volume 1 of the first addition (as indicated above) are incorporated in the phylum Proteobacteria. Volume Two, Part B contains representatives from the class **Gammaproteobacteria**, and Volume Two, Part C contains representatives from the classes **Alpha**, **Beta**, **Delta** and **Epsilonproteobacteria**. Volume 3 of the newest edition includes Gram-positive organisms categorized within the phylum **Firmicutes**. This phylum includes Gram-positive spore-formers (*Clostridium*, *Bacillus* and multiple new *Bacillus*-like genera), and non-sporing genera including *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Staphylococcus, Exiguobacteium, Macrococcus, Salinicoccus, Planomicrobium* and *Leuconostoc*. Other volumes have yet to be published.

**Enrichment Procedures For Some Selected Organisms From Soil**

**Enrichment procedures** involve the use of media or techniques specifically designed to promote the growth of certain organisms while inhibiting the growth of others. When applied to diverse microbial **communities** enrichments allow specific **populations** (species of organisms) to be preferentially propagated. These can then be isolated or separated from one another through the application of pure culture techniques.

Soil provides a habitat rich in microbial diversity. A single gram of soil, depending on nutrient load, may contain a million or more cells. Since most of the organisms living in soil are bacteria, soil is an excellent source of material for enrichment procedures. Three bacteria genera readily isolated from soil through enrichment include ***Pseudomonas***, ***Azotobacter*** and ***Bacillus***. Of these, *Pseudomonas* and *Azotobacter* include respiratory, Gram-negative forms (phylum **Proteobacteria**), and *Bacillus* includes Gram-positive, endospore-forming organisms (phylum **Firmicutes**). Individual species from these three genera can easily be isolated from a single sample of soil as indicated below.

**Genus I – *Pseudomonas***

Bacteria in the genus *Pseudomonas* are primarily aerobic organisms inhabiting soil and water; a few are pathogenic to humans. They are typically motile by polar flagella, and in culture may become "swarmers". They produce a variety of non-photosynthetic pigments (some of which are fluorescent) and are sometimes accompanied by a characteristic odor.

One of the most remarkable physiological properties of the genus *Pseudomonas* is the very wide range of unusual organic compounds they can use as sources of carbon and energy. It has been shown that over 90 different organic substrates can be oxidized by a single strain of *Pseudomonas fluorescens*. In at least some species, the utilization of unusual carbon sources is made possible by genes associated with plasmids.

**Enrichment Procedure for *Pseudomonas***

The enrichment medium for *Pseudomonas* provides **sodium benzoate** as the only carbon source. Sodium benzoate is an organic compound commonly used as a preservative in foods and beverages because it is toxic to most microorganisms. It is not a carbon source utilized by many types of bacteria. Since several species of *Pseudomonas* can use this compound, it forms the basis for an effective enrichment.

**Genus II – *Azotobacter***

Bacteria in the genus *Azotobacter* are free-living inhabitants of soil, water, and the surfaces of leaves. They are typically larger than other Proteobacteria, often forming cells greater than 2µm in diameter. Many species form capsules or slime layers making their colonies appear wet (sometimes dripping into plate lids) when grown on solid media.

A unique feature of bacteria in the genus *Azotobacter*, is their ability to "fix" atmospheric nitrogen, i.e., convert molecular nitrogen ($N_2$) from the atmosphere into ammonia ($NH_3$), nitrate ($NO_3^-$), or nitrite ($NO_2^-$), thus making nitrogen available to plants and other organisms.

**Enrichment procedure for *Azotobacter*:**

The enrichment medium for *Azotobacter* contains no nitrogen, thus only bacteria capable of fixing nitrogen from the atmosphere can grow in it. Only small amounts of fixed nitrogen, not enough to support the growth of muliple cells, will be added during the inoculation of the medium.

Members of the genus *Azotobacter* are not the only bacteria capable of growing in nitrogen-free media. Although the medium used for this enrichment was designed to promote the growth of *Azotobacter*, it will also support the growth of other nitrogen-fixing genera. The *Azotobacter* species can be distinguished from most other genera by their larger size.

**Genus III – Bacillus**

Organisms in the genus *Bacillus* belong to the family Bacillaceae, a family including endospore-forming rods often motile by peritrichous flagella, and usually staining Gram-positive. Endospore forming bacteria may be easily separated from other species by taking advantage of the greater heat resistance of their endospores. The vegetative (non-sporing) cells of most organisms cannot survive being heated to temperatures above 60-70$^o$ C because their proteins are denatured at these temperatures (thermophilic bacteria are an exception). During endospore formation, the proteins within a sporangium apparently change in such a way that they are no longer so readily denatured, and higher temperatures can be endured. This is especially true of thoroughly dried spores (exospores).

Because the endospores formed by *Bacillus* and other endospore-forming genera are thermoduric, an effective enrichment for these organisms involves **pasteurization**.

Pasteurization, a heat treatment developed by **Louis Pasteur** in 1862, will effectively kill the vegetative cells of most bacteria, eliminating them from the enrichment sample. The time required to kill depends on the temperature to which the cells are subjected. One minute at 100$^o$ C, 5 minutes at 80$^o$ C, 10 minutes at 70$^o$ C, or 20 minutes at 60$^o$ C are times commonly employed for pasteurization. Once vegetative cells have been removed, the enrichment sample can be inoculated onto nutrient agar and the spores will germinate, forming new cells capable of colony development.

**Procedure:**

**A. Enrichment for *Pseudomonas* (Initiated during Exercise 4)**

1. Examine the *Pseudomonas* enrichment medium (broth tube) inoculated during Exercise 4 and incubated at room temperature on the orbital shaker. Look for the presence of growth; as indicated by increased turbidity and bubble formation.

2. Aseptically streak a loopful of the enrichment culture onto a plate of sodium benzoate agar. Incubate the plate at room temperature in your lab drawer until the next laboratory period.

3. Examine the sodium benzoate plate for colonies of Gram-negative rods by preparing a Gram stain from one or more of the colonies present. Be careful to practice aseptic technique while working with this plate, as it might be useful as a source of organisms for a semester project.

**Note** - To isolate and identify a pure culture of *Pseudomonas* as a semester project, select a well-isolated colony from the sodium benzoate plate and obtain a pure culture by streaking a new plate of nutrient agar (NA), Tryptic Soy agar (TSA) or Mueller-Hinton agar (MHA). Any contaminant cells carried along with the *Pseudomonas* culture are likely to grow on these media and become apparent. If the new streak plate shows only one type of colony composed of Gram-negative, motile rods, you can assume the culture is pure and proceed with your identification.

**B. Enrichment for *Azotobacter* (Initiated during Exercise 4)**

1. Examine the *Azotobacter* enrichment medium (broth tube) inoculated during Exercise 4 and incubated at room temperature on the orbital shaker. Look for the presence of growth; as indicated by increased turbidity and bubble formation.

2. Aseptically streak a loopful of the enrichment culture onto a plate of nitrogen-free agar medium. Incubate the plate at room temperature in your lab drawer until the next laboratory period.

3. Examine the nitrogen-free agar plate for colonies of *Azotobacter*. These develop as large glistening mucoid white or transparent ovals that often turn dark brown or black with age. When your plate develops colonies of this type, prepare a Gram stain and look for large, Gram-negative coccobacilli. You may wish to prepare a capsule stain of *Azotobacter* as instructed in a previous exercise, since *Azotobacter* species often form large capsules. Be careful to practice aseptic technique while working with this plate, as it might be useful as a source of organisms for a semester project.

**Note** - To isolate and identify a pure culture of *Azotobacter* as a semester project, restreak a selected colony onto a slant of *Azotobacter* medium, and then check the purity of the slant culture by streaking a plate of nutrient agar. Any contaminant cells carried along with the *Azotobacter* will be likely to grow on the nutrient medium and become apparent. If a streak plate of nutrient agar shows only one type of colony composed of large, Gram-negative coccobacilli, you can assume the culture is pure and proceed with your identification. There are multiple other genera of bacteria able to grow on nitrogen-free medium. If the colonies contain cells less than 2mm in diameter, they are probably not *Azotobacter*.

### C. Enrichment for *Bacillus* (Initiated during Exercise 6)

1. Place 1 gram of soil in a test tube and wash it into the bottom with about 5 ml of distilled water. Do not leave soil on the sides of the tube since soil there may not be subjected to the measured temperature.

2. Place the tube in a beaker of boiling water, let it stand for 1 minute and then remove it and cool it quickly by running cool tap water over the outside.

3. Obtain a plate of nutrient agar. The surface of the plate should be dry to prevent motile species of *Bacillus* from spreading so rapidly across the surface that it is impossible to obtain discrete colonies.

4. Streak the surface of the plate with a loopful of liquid from the pasteurized soil suspension (do not streak the plate with a loopful of mud). **Remember** to use a streak technique likely to yield well-isolated colonies.

5. Incubate the plate at room temperature until the next laboratory period and at that time examine it for colonies.

6. Prepare Gram stains of two or more different looking colonies and observe them under oil immersion. Look for endospores and note their shape and location. In a Gram stain preparation endospores will not usually take up stain and so will appear as white or pale-colored spots within a dark colored cell. Be careful to practice aseptic technique while working with this plate, as it might be useful as a source of organisms for a semester project.

**Note** - To isolate and identify a pure culture of *Bacillus* as a semester project, restreak a selected colony onto a fresh plate of nutrient agar. If this streak plate shows only one type of colony composed of large, Gram-positive, endospore-forming bacilli, you can assume the culture is pure and proceed with your identification. Since the genus *Bacillus* has recently been divided into multiple new genera (*Lysinibacillus*, *Paenibacillus*, *Brevibacillus*, etc.), your endospore-forming isolate may turn out to be one of these.

### Enrichment Procedure for Luminous Bacteria

Several species of bacteria in the family Vibrionaceae (Genera *Vibrio* and *Photobacterium*) appear to glow (emit light) when their populations are observed in the dark. Such organisms are referred to as **luminous** or **bioluminescent** and often form symbiotic relationships with marine animals including deep-water fish and squids. Luminous bacteria are able to convert chemical energy into light energy via the activity of enzymes called **luciferase enzymes**. The metabolic pathways involved are energy "expensive", but are believed to give luminous bacteria a survival advantage in their natural habitat.

Enrichment procedures for luminous bacteria involve media made with seawater or salt solutions having a similar composition. Samples taken from freshly killed fish or squid can be streaked on such media and incubated at room temperature or in the refrigerator. Since many luminous bacteria are **psychrophilic** and grow slowly, plates must be observed over several days. The period of optimum light production is usually limited to a few hours. Investigators must work in the dark and allow their eyes time to adjust to low light conditions. When luminous colonies are observed, they can be selected from among the other species present and transferred to new media.

**Procedure:**

1. Using a sterile cotton swab, obtain a sample of intestinal contents from the animal selected (fish or squid).

2. Use the swab to inoculate about 1/4 of a luminous medium plate and then use a sterile loop to dilute the culture using a typical streak technique. Let the swabbed area represent your first streak area and then streak areas 2 and 3 with a sterile loop.

3. Incubate the plate in the refrigerator or at room temperature for 2-3 days. When growth is evident, take the plate into a dark room and look for luminous colonies. Remember to allow your eyes time to adjust to the dark. The light emitted by a single colony of luminous bacteria may not be very bright.

4. If luminous colonies are present, try to touch each of them (one at a time) with a sterile loop and transfer samples to a new plate of luminous medium. It is not necessary to streak a separate plate for each colony, since several samples can be grown on a single plate.

5. Incubate the new plate and again examine for luminous colonies. If one or more of the individual samples show luminescence, colonies from that area can be streaked on a new plate for isolation. When all of the colonies growing on your streak plate show a similar degree of luminescence, you can assume you have obtained a pure culture.

**Note** – To isolate and identify a pure culture of luminous bacteria as a semester project, you can restreak a selected colony onto a fresh plate of luminous agar. If this streak plate shows only one type of colony and all colonies show a similar degree of luminescence, you can assume the culture is pure and proceed with your identification.

**Isolation of Some Selected Organisms from Air**

Although air contains little or no nutrients, and does not usually support the growth of microorganisms, many types of bacteria are present in air. Common sources of air-borne bacteria include soil, water, the epithelial surfaces of animals and plants, saliva and respiratory secretions. Many genera of Gram-positive bacteria can be easily isolated from air, including *Micrococcus*, *Deinococcus*, *Kocuria*, *Kytococcus*, *Microbacterium*, *Arthrobacter* and *Bacillus*. Most species of the first six genera form spherical-shaped cells or coccobacilli, are usually non-motile, and often form pigments (typically yellow, orange, red or pink). They usually form circular, entire, smooth-shiny, slightly convex, opaque or semi-translucent colonies on nutrient agar. *Bacillus* and related species are also common on air plates, and typically form circular to irregular, undulate, rough-textured, raised, opaque colonies on nutrient agar. Some are filamentous and spread rapidly across the agar surface. Multiple genera of Gram-negative bacteria can also grow on air plates. Samples from colonies present on air plates can be restreaked onto new nutrient agar plates and used as a source of organisms for semester projects.

**Procedure:**

1. Observe the morphological features of the colonies present on the air plate you prepared during an earlier exercise.

2. Make a Gram stain from one or more of the colonies and observe the cells under oil immersion. Record their appearance.

3. Select a well-isolated colony of interest, and streak a sample from this colony onto a new nutrient agar plate. If this plate shows only one type of colony, and all the cells are similar, you can assume the culture is pure and proceed with your identification.

# Exercise 7-B
# INTRODUCTION TO CYANOBACTERIA

## Introduction

The **cyanobacteria**, currently representing a single phylum in the domain bacteria, are often considered unlike other bacteria. Until the 1960's they were classified as plants in the division cyanophyceae and were commonly referred to as blue-green algae. In the eighth edition of the *Bergey's Manual of Determinative Bacteriology* they were listed within a separate division, and in the first edition of the *Bergey's Manual of Systematic Bacteriology* they were included as bacteria with unusual properties (**oxygenic phototrophic bacteria)** in Volume 3. The cyanobacteria are aerobic, photosynthetic organisms with Gram-negative cell walls. They may occur as cocci, rods, or spirilla, are widely distributed in water (fresh and salty), and are common in damp soil. Different species may appear as blue-green, green, yellow, red, purple, black, or colorless.

The cell walls of cyanobacteria often have a much thicker outer membrane than those of other bacteria. Many forms have an “envelope" outside this wall, variously referred to as a **sheath**, a **capsule**, or a **slime layer**. **Fimbriae** are numerous on some cells, but none of them have flagella. Motile forms demonstrate a "gliding" motion, the mechanism for which is not thoroughly understood. Individual cells of cyanobacteria vary considerably in size (from 1 to more than 100 microns in diameter) and are often arranged in complexes causing them to appear macroscopic. Many cyanobacteria form short or long chains of cells called **trichomes.** These may be interrupted by pale-colored, thick walled bodies called **heterocysts** in nitrogen-fixing forms, or by "resting spores" called **akinetes**. The **terminal cells** of trichomes are often modified into unusual shapes and sometimes elongated into **hairs**. The classification of cyanobacteria was initially based primarily on morphology, with freshwater forms being more thoroughly studied than marine varieties.

## Procedure:

A. Observe the prepared slides of cyanobacteria listed below. Be able to recognize and identify the structures indicated in the attached diagram.

   1. *Oscillatoria*

   2. *Nostoc*

   3. *Gloeocapsa*

   4. *Anabaena*

   5. *Spirulina*

B. Prepare wet mounts of and observe the living specimens of the organisms provided. Note the gliding motion characteristic of some cyanobacteria. Be able to identify the following:

1. *Oscillatoria*

2. *Gloeocapsa*

3. *Nostoc*

4. *Anabaena*

5. *Spirulina*

C. Prepare wet mounts from the natural infusions present and try to identify some of the organisms present.

**<u>Questions:</u>**

1. What is an enrichment procedure or medium?

2. Can you explain the enrichment procedures used in this exercise to culture three different genera of bacteria from a single soil sample? (Be able to!)

3. Were members of the genus *Azotobacter* the only types of bacteria observed to be growing on the nitrogen-free medium?

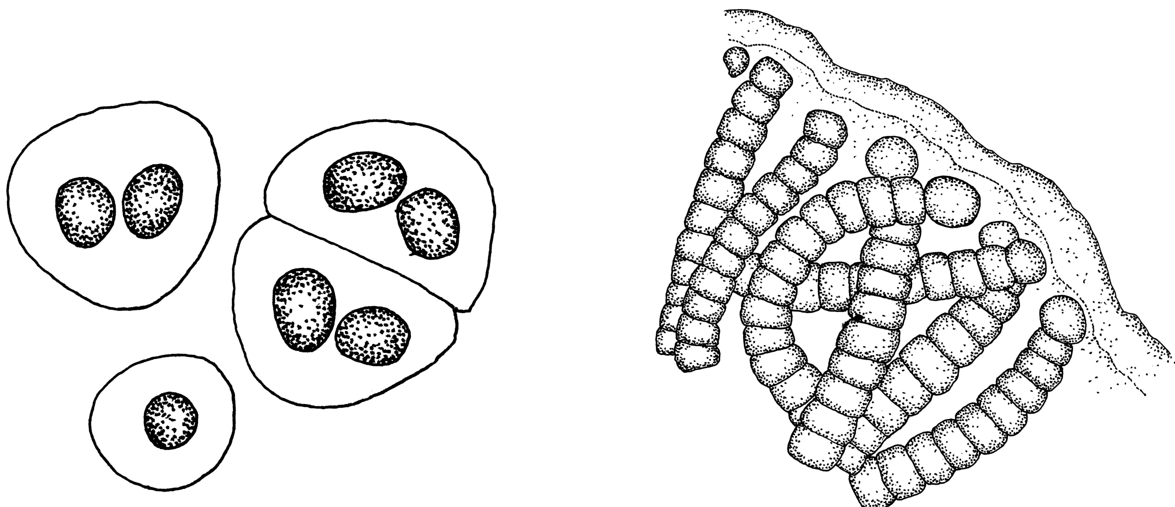
4. Do you think *Pseudomonas*, *Azotobacter* and *Bacillus* were the only types of bacteria present in your soil sample? Why or why not?

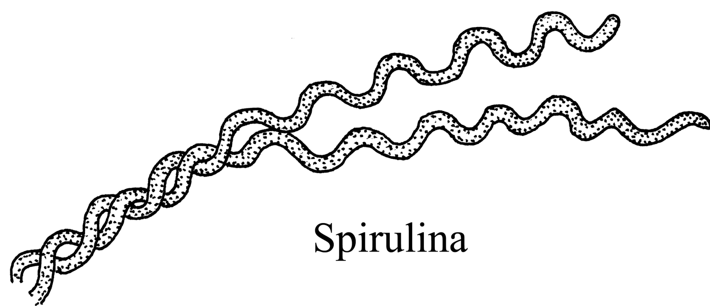
5. What is bioluminescence and what enzymes are associated with this phenomenon?

6. How do the cyanobacteria differ from most of the eubacteria you have been working with in lab?

7. How do the cyanobacteria differ from eukaryotic algae?

8. Do any of the cyanobacteria you observed display motility? Do they possess flagella?



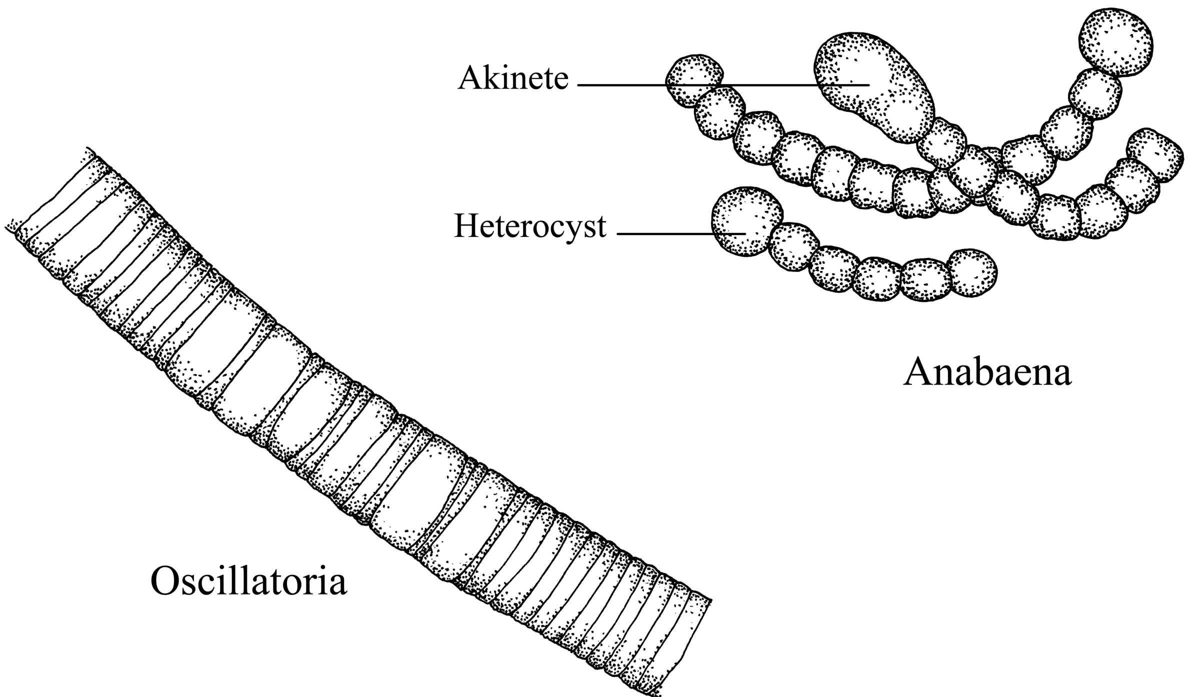




**Fig. 7-B.1 - Some representative examples of common cyanobacteria.**

NOTES, OBSERVATIONS & ADDITIONAL INFORMATION