Chapter 4

Cultural, Physiological, and Biochemical Identification of Actinobacteria

Qinyuan Li, Xiu Chen, Yi Jiang and Chenglin Jiang

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Abstract

The traditional phenotypic tests are commonly used in actinobacterial identification. They constitute the basis for the formal description of taxa, from species and subspecies up to genus and family. The classical phenotypic characteristics of actinobacteria comprise morphological, physiological, and biochemical features. The morphology of actinobacteria includes both cellular and colonial characters. The physiological and biochemical features include data on growth at different temperatures, pH values, salt concentrations, or atmospheric conditions, and data on growth in the presence of various substances such as antimicrobial agents, the presence or activity of various enzymes, and with respect to metabolization of compounds. The phenotype is the observable expression of the genotype. Gene expression is directly related to the environmental conditions. Actinobacterial phenotype cannot be based on the simple observation of the organism. Strains of the most closely related taxa should be compared in their phenotypic analysis using identical methods. The comparisons must include the type strain of the type species of the appropriate genera. Furthermore, with the development of technology, microbial physiological and biochemical identification technology is becoming fast, simple, and automated.

Keywords: Phenotype, Physiological and biochemical characteristics, Automatic identification system

1. Introduction

The polyphasic approach [1], the comprehensive results of various methods, such as morphological, physiological, rRNA gene sequencing, chemotaxonomic markers and pathogenicity,

are used to compile a description of a new species. Classification of actinobacteria is now based largely on analysis of the nucleotide sequences, especially 16S rRNA genes [2]. However, a comprehensive characterization of a new species should still be identified, because from even a complete genomic sequence, it would be difficult to predict many of the phenotypic features of a new species [3]. Physiological and biochemical characteristics are directly related to the activity of microbial enzymes and regulatory proteins. Enzymes and proteins are gene products; so the comparison on physiological and biochemical characteristics of actinobacteria is the indirect comparison of genome, and determination of physiological and biochemical characteristics is much easier than direct analysis of the genome. Therefore, the physiological and biochemical characteristics in actinobacterial systematics and identification are still meaningful [4]. In addition to a thorough phenotypic characterization of a new species, it is important to determine which phenotypic features are the ones most useful for identifying new species. Moreover, the particular methods used for characterizing an organism should always be stated, because the results of phenotypic tests can vary with methodology.

Phenotypic analysis is a very tedious task in the classification of actinobacteria. The classical phenotypic characteristics of actinobacteria comprise morphological, physiological, and biochemical features. Individually sufficient as parameters for genetic relatedness, yet as a whole, they provide descriptive information enabling us to recognize taxa [5]. The morphological traits include both cellular (cell shape and size, spore, sporangia, sporangiospore, the location of the spores or sporangia and their size, flagella, motility, intracellular structures, etc.) and colonial characters (shape and size, color, dimensions, form, etc.). The physiological and biochemical traits include data on growth at different temperatures, pH values, salt concentrations, atmospheric conditions (aerobic/anaerobic), growth in the presence of various substances such as antimicrobial agents, and data on the presence or activity of various enzymes, metabolization of compounds, and so on (Table 1). Physiological and biochemical tests should be carried out in test media and under conditions that are identically standard or at least comparable. There are three major inter-related areas in actinobacterial identification: taxonomic relevance; methodological reliability and cost effectiveness; and data portability [6]. It must be noted that novel taxa should be described based on the characteristics of more than one related strain and the type strain of the type species of the appropriate genera [7]. It should be emphasized that some phenotypes are encoded by extrachromosomal inheritance factors and the influencing factors that affect the expression of physiological and biochemical traits are complicated. To determine the genetic relationships based on physiological and biochemical characteristics,systematic classification, must be integrated with other characteristics, particularly genotype characteristics analysis.

Identification of a species is a constant basic work in any microbiology laboratory. Regardless of the type of microorganisms, the working steps are inseparable from the following three items: (i) to obtain the pure cultures of microorganism, (ii) to determinate the necessary appraisal indicators, (iii) to find authoritative identification manuals and publications, and related site information. Different organisms often have their own different identification priorities. For example, the identification of microorganisms with rich morphological characters, such as fungi, often bases on their morphological features as the main indicators; the

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identification of actinobacteria and yeasts, synthesizes the morphological, physiological and biochemical characteristics; the identification of bacteria lacking morphological difference, often uses more physiological, biochemical, and genetic parameters.

Characteristics	The difference between groups
Adaptability of temperature	The optimal, the lowest, and highest growth temperature and die temperature
Adaptability of pH values	The range of pH values at which it can grow, as well as the optimal pH for growth
Adaptability of osmotic pressure	The salt concentration and halophilism
Utilization of nitrogen source	Utilization of proteins, peptone, amino acids, nitrogen, inorganic salt, N_2, etc.
Utilization of carbon source and acid-producing ability	Utilization of various monosaccharides, disaccharides, polysaccharides, alcohols, and organic acids, etc. Acid production from carbohydrates
Needs of growth factors	Special vitamins, amino acids, X and V factor requirements
Atmospheric condition	Aerobic, microaerophilic, anaerobic, facultative anaerobic
Antimicrobial activity	Inhibition to Gram-positive and Gram-negative bacteria, filamentous fungi and yeast, etc.
Metabolization	Various characteristic metabolites tests, such as MR test, V-P test, iodole production, etc.
Activity of various enzymes	Oxidase, catalase, urease, etc.
Sensitivity	The sensitivity to antibiotics, potassium cyanide (potassium sodium), antimicrobial agents, dyes, etc.

Table 1. Common physiological and biochemical characteristics used for classification and identification of actinobacteria

2. Cultural characteristics of actinobacteria

Cultural characteristics of actinobacteria refer to the growth characteristics and morphology in various kinds of culture media. Pure culture should be taken before morphological observation. The pure culture of actinobacteria can be obtained through the use of spread plates, streak plates, or pour plates and are required for the careful study of an individual microbial species [Figure 1]. Cultural characteristics on 4 to 6 media are usually determined after incubation 14 to 28 days at 28°C strictly according to the methods used in the *International Streptomyces Project* (ISP) [8]. Sometimes, other media can be chosen, such as nutrient agar and czapek's agar. The colors of substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from the ISCC-NBS color charts [9].

Nutrient agar medium	**(G/Liter)**
Peptone	10.0 g
Beef extract/yeast extract	3.0 g
NaCl	5.0 g
Agar	15.0 g
Final pH (at 25°C)	7.0±0.2
Czapek's agar medium	**(G/Liter)**
Sucrose	30.0 g
$NaNO_3$	3.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 4H_2O$	0.01 g
K_2HPO_4	1.0 g
Agar	15.0 g
Final pH (at 25°C)	7.2±0.2

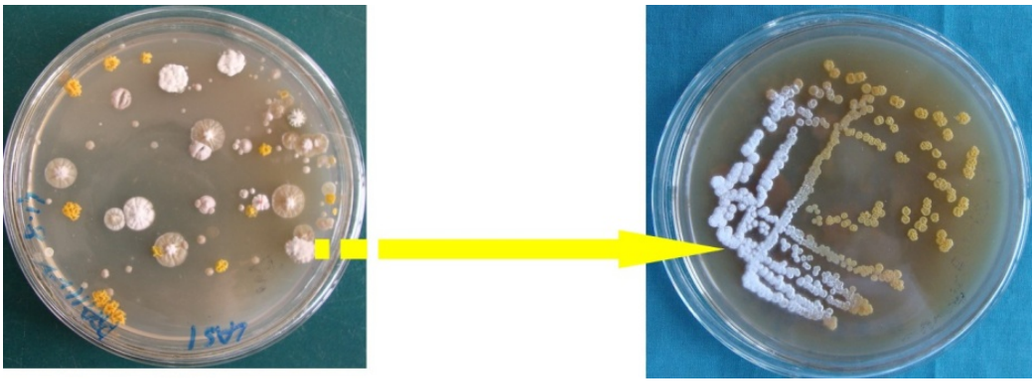

Figure 1. Acquisition of pure culture

3. Physiological and biochemical characteristics for identification of actinobacteria

Some phenotypic characteristics of actinobacteria are of such primary importance to a genus or species description. Several problems must be considered when planning the physiological and biochemical tests of actinobacteria. One of them, according to the phylogenic information based on 16S rRNA analyses, strains of the most closely related taxa and the type strain of the type species of the appropriate genera should be chosen for comparison in their phenotypic traits. Other problems are concerned with methodology. In classifying actinobacteria, it is desirable to use an established approach based on common sense, and to use tests that are pertinent. If novel methods are used, the researcher must provide evidence that the new methods produce comparable results to established methods. Furthermore, phenotypic

characteristics of actinobacteria are influenced by cultural conditions and other factors, so tests should be performed in duplicate or triplicate. More importantly, design reasonable positive and negative controls in the experiments.

3.1. Temperature range and optima for growth

Incubate cultures at a range of temperatures; using constant temperature incubators or water baths, measure the growth response of the actinobacteria. The tested temperature range is usually from 0°C to 75°C. In general, temperature experiments employ solid medium instead of broth in order to better observe. The basal medium is Bennett's medium or YIM38 medium or nutrient medium.

Bennett's medium	**(G/Liter)**
Yeast extract	1.0 g
Beef extract	1.0 g
Casein	2.0 g
Glucose	10.0 g
Agar	15.0 g
Final pH (at 25°C)	7.3±0.2
YIM 38 medium	**(G/Liter)**
Malt extract	10.0 g
Yeast extract	4.0 g
Glucose	4.0 g

Vitamin mixture (0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxine-HCl, inositol, calcium pantothenate, and p-aminobenzoic acid and 0.25 mg biotin)

Agar	15.0 g
Final pH (at 25°C)	7.2±0.2

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Mix well and pour into sterile Petri plates.

Note: If the temperature is above 100°C, use screw-cap culture tubes or screw-cap glass bottles and seal the screw caps to prevent the evaporation of the medium. Incubate the cultures in commercial water baths filled with dimethyl silicone oil. If the temperature is below 0°C, use an ethylene glycol water bath. For marine actinobacteria that require seawater, open ocean seawater has NaCl concentration of about 35 g/L or 3.5% (wt/vol).

3.2. Optimum pH and pH range for growth

An essential part of the description of any actinobacteria is the range of pH values at which it can grow, as well as the optimal pH for growth. Measure growth responses from a standar-

dized inoculum using basic medium (Bennett's medium or YIM38 medium or nutrient medium) at various pH values. Liquid medium is to be used for pH tests, to measure the growth responses turbidimetrically. The selection of buffer is critical. A buffer should be used in most media to maintain a stable pH for growth of the test strain. Buffers are most effective at their pK_a values and should be chosen with this in mind. Some useful biological buffers are listed in Table 2. Some buffers such as citrate, succinate, or glycine may be metabolized by the test organism. Others may be toxic. Sometimes, a combination of buffers may be helpful. Certain buffers (Good buffer) are non-metabolizable, non-toxic, have low reactivity with metal ions, and have other desirable features [10]. Phosphate salts are most commonly used because they are effective in the growth range of most bacteria, are usually non-toxic, and provide a source of phosphorus for the organism.

Buffer	**Effective pH range**	**pK_a (25°C)**
Maleate (Salt of maleic acid)	1.2–2.6	1.97 (pK_a1)
Phosphate (Salt of phosphoric acid)	1.7–2.9	2.15 (pK_a1)
Glycine	2.2–3.6	2.35 (pK_a1)
Citrate (Salt of citric acid)	2.2–3.5	3.13 (pK_a1)
Malate (Salt of malic acid)	2.7–4.2	3.40 (pK_a1)
Citrate (Salt of citric acid)	3.0–6.2	4.76 (pK_a2)
Succinate (Salt of succinic acid)	3.2–5.2	4.21 (pK_a1)
Acetate (Salt of acetic acid)	2.6–5.6	4.76
Malate (Salt of malic acid)	4.0–6.0	5.13 (pK_a2)
Succinate (Salt of succinic acid)	5.5–6.5	5.64 (pK_a2)
MES 2-(*N*-Morpholino)-ethanesulfonic acid	5.5–6.7	6.10
Maleate (Salt of maleic acid)	5.5–7.2	6.24 (pK_a2)
Citrate (Salt of citric acid)	5.5–7.2	6.40 (pK_a3)
ACES (*N*-(2-Acetamido)-aminoethanesulfonic acid)	6.1–7.5	6.78
BES (*N*,*N*-Bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid)	6.4–7.8	7.09
MOPS (3-(*N*-Morpholino)-propanesulfonic acid)	6.5–7.9	7.14
HEPES (*N*-(2-Hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid)	6.8–8.2	7.48
Phosphate (Salt of phosphoric acid)	5.8–8.0	7.20 (pK_a2)
Imidazole	6.2–7.8	6.95
TES (2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid)	6.8–8.2	7.40
Tricine (N-[Tris(hydroxymethyl)-methyl]-glycine)	7.4–8.8	8.05
Tris (Tris(hydroxymethyl)-aminomethane)	7.5–9.0	8.06
TABS (*N*-tris[hydroxymethyl]-4-amino-butanesulfonic acid)	8.2–9.6	8.90
CHES (Cyclohexylaminoethanesulfonic acid)	8.6–10.0	9.50
Glycine	8.8–10.6	9.78 (pK_a2)
CAPSO (3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid)	8.9–10.3	9.60
CAPS (3-(Cyclohexylamino)-propanesulfonic acid)	9.7–11.1	10.40

Table 2. Common biological buffers, their effective range, and their p*Ka* values at 25°C

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Usually, it is necessary to test the growth of actinobacteria from pH 4.0 to 13.0 and determine the strain growth pH range and the optimum pH value by using the following buffer system: pH 4.0–5.0: 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0: 0.1 M KH_2PO_4/0.1 M NaOH; pH 9.0–10.0: 0.1 M $NaHCO_3$/0.1 M Na_2CO_3; pH 11.0: 0.05 M Na_2HPO_4/0.1 M NaOH; pH 12.0–13.0: 0.2 M KCl/0.2 M NaOH [11]. Negative controls for each buffer were used and the final pH was determined by using an indicator of acidity.

3.3. NaCl ranges and optima for growth

Salt tolerance experiments mainly test the tolerance ability of the organism to NaCl and other salts, and determine the optimum concentration for growth. Inoculate liquid media containing a range of NaCl (usually 0–30%, W/V, or relative molar concentrations) concentrations and measure the growth response turbidimetrically. Bennett's medium or YIM38 medium or nutrient medium can be used as basal medium.

For some marine actinobacteria, NaCl alone may not substitute for filtered seawater, which should be sterilized by filtration and added aseptically to the sterilized medium. Even if seawater is used, it may need to be aged for a few weeks in a glass vessel in the dark to be effective. Seawater contains 3% NaCl, and testing marine organisms for growth at levels below this can be done by using various proportions of distilled water to seawater in the medium, or by using artificial seawater in which the level of NaCl can be varied.

3.4. Utilization of carbon source

Utilization of carbon source tests usually uses turbidimetric method. Use a chemically defined basal medium that lacks a carbon source, but otherwise is suitable for growth of the actinobacteria being tested. The basal medium is Pridham and Gottlieb carbon utilization medium [8]. Add carbon sources to a concentration (sugar alcohols 0.5–1%, others 0.1–0.2%). After growth has occurred, measure the growth response turbidimetrically with a spectrophotometer.

Basic medium for carbon source utilization	**(G/Liter)**
$(NH_4)_2SO_4$	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4 \cdot 7H_2O$	1.0 g
$CuSO_4 \cdot 5H_2O$	0.0064 g
$FeSO_4 \cdot 7H_2O$	0.0011 g
$MnCl_2 \cdot 4H_2O$	0.0079 g
$ZnSO_4 \cdot 7H_2O$	0.0015 g
Final pH (at 25°C)	7.2–7.4

Note:

1. Use liquid medium to avoid the influence of agar.
2. Thermolabile carbon sources should be sterilized by filtration (filter sterilize 10% solution through bacteriological filter) or ether sterilization (weigh an appropriate amount of the dry carbon source and spread in a pre-sterilized Erlenmeyer flask fitted with a loose cotton plug. Add sufficient acetone-free ethyl ether to cover the carbohydrate. Allow ether to evaporate at room temperature under a ventilated fume hood overnight or longer. When all ether has evaporated, add sterile distilled water aseptically to make a 10% w/v solution of the carbon source).
3. Controls required for the test: No carbon source (negative control); D-glucose (positive control).
4. For marine actinobacteria, instead of distilled water, use a synthetic seawater and sterilize the media by filtration.

3.5. Utilization of nitrogen source

Use the turbidimetric method to test the utilization of nitrogen source, especially sole nitrogen sources. Basal medium that omit nitrogen source but include a suitable carbon source are used. Add nitrogen source to a concentration (usually 0.5%).

Basic medium for nitrogen source utilization	**(G/Liter)**
D-Glucose	1.0 g
$MgSO_4 \cdot 7H_2O$	0.05 g
NaCl	0.05 g
$FeSO_4 \cdot 7H_2O$	0.001 g
K_2HPO_4	0.01 g
Final pH (at 25°C)	7.2–7.4

Note:

1. Use liquid medium to avoid the influence of agar.
2. Thermolabile nitrogen sources should be sterilized by filtration or ether sterilization.
3. Controls required for the test: negative control (no carbon source).

3.6. Enzymological characteristics

Some enzymological characteristics are of such primary importance to a genus or species description that they must appear in every published description.

Oxidase test

This method tests for an enzyme that transfers electrons from a donor molecule to O_2, thereby forming H_2O. Oxidase-positive organisms are usually aerobes or microaerophiles that can use O_2 as their final electron acceptor. The test reagent, N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), acts as an artificial electron acceptor for the oxidase and the reduced form is the colored compound indophenol blue.

Prepare a 1% (wt/vol) solution of TMPD in certified-grade dimethylsulfoxide (DMSO). The solution is stable for at least a month under refrigeration. Test methods:

a. Method described by Kovacs [12]: Soak a small piece of filter paper in 1% Kovács oxidase reagent and let dry. Use a loop and pick a well-isolated colony from a fresh (18- to 24-h culture) bacterial plate and rub onto treated filter paper (please note on recommended media and loops). Observe for color changes. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 sec. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 sec. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 min.

b. Method of Tarrand and Gröschel [13]: Place a piece of Whatman no. 40 ashless filter paper, quantitative grade, in a petri dish and wet it with 0.5 ml of the TMPD-DMSO solution. Use a cotton-tipped swab to pick up one large isolated colony and allow the inoculum on the swab to dry for 5 sec. Tamp the swab lightly 10 times on the wet filter paper. Development of a blue-purple color in 15 sec is a positive test.

c. Colony methods: 1% TMPD directly drops on the colonies. The color from pink to scarlet in 15 sec is a positive test. To speed up the color change, add one drop of toluene first on colonies.

Note:

1. For this test, do not use cultures grown on selective media. Results from old cultures may be unreliable. Use the organism in exponential growth period. Do not use cultures grown on media containing fermentable carbohydrates, as acid from fermentation may inhibit oxidase enzyme activity and result in false negatives.

2. This test should avoid iron pollution, otherwise easy to produce false positive results. Reagent is fresh.

3. Reagent is highly toxic; contact with skin should be avoided.

Catalase test

Catalase catalyzes the disproportionation reaction $2H_2O_2 \rightarrow 2H_2O + O_2$, thereby helping to prevent oxidative damage to cells caused by H_2O_2. Add 0.2 ml of a 3–10% H_2O_2 solution to a screw-cap test tube. Using a platinum loop, disposable plastic loop, or glass rod, remove some growth from a colony or agar slant and rub the growth on the inner wall of the tube. Cap the tube (to prevent escape of aerosols) and slant it, so that the H_2O_2 solution covers the growth. Effervescence within 30 sec indicates a positive reaction.

Note: For this test, do not use cultures grown on blood-containing media, as blood contains catalase; however, cultures grown on a medium containing heated blood, such as chocolate agar, can be used. Some bacteria can make catalase only when provided with heme; these organisms are negative when cultured on media lacking blood, but are positive when cultured on chocolate agar. Some bacteria make a pseudocatalase (a non-heme catalase) when grown on media lacking blood, but containing little or no glucose; they are negative for catalase when cultured on media containing 1% glucose. With anaerobic organisms, expose the culture to air for 30 min before performing the test, as some anaerobes have an inducible catalase.

Urease test

Urease test check he ability of an organism to produce an exoenzyme, called urease. Urease catalyzes the reaction $(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$. The ammonia that is formed causes the medium to become alkaline: $NH_3 + H_2O \rightarrow NH_4^+ + OH^-$. The alkalinity can be detected with a pH indicator.

Prepare the following medium (per liter of distilled water): peptone 1 g; NaCl 5 g; glucose 1 g; KH_2PO_4 2 g; phenol red 0.012 g; agar 15 g; pH 6.8–6.9. Autoclave and cool to 55°C. Add 30% (W/V) filter-sterilized (or ether-sterilized) solution of urea to make final concentration of 2%. Mix and dispense appropriate portions into tubes or plates. Prepare a control medium lacking urea. Inoculate the surface of the slant or plate for 4 days. Look for development of a red-violet color compared to an uninoculated control. The medium can also be prepared as a liquid medium by omitting the agar [14].

For other organisms that grow poorly or not at all on Christensen's medium, use a test medium of the composition (per liter of distilled water): BES buffer 1.065 g; urea 20.0 g; phenol red 0.01 g; pH 7.0. Also prepare a control medium lacking urea. Sterilize both media by filtration and dispense 2.0-ml portions into sterile tubes. Culture the test organism in a suitable liquid medium. Centrifuge the cells and suspend them in sterile distilled water to a dense concentration. Add 0.5 ml of the suspension to the test medium and the control medium. Incubate the tubes for 24 h at the optimal temperature for the organism. Look for the development of a red-violet color in the test medium, but not in the control medium [15].

Lipase test

Lipase activity can be shown by using Tweens, for example, Tween 80 (polyethylene sorbitan monooleate, an oleic acid ester), Tween 40 (a palmitic acid ester), and Tween 20 (a stearic acid ester). Lipolytic organisms split off the fatty acid, and the calcium salts of the fatty acids produce opaque zones around the colonies.

Prepare a basal medium containing the following (per liter of distilled water): peptone 10.0 g; NaCl 5.0 g; $CaCl_2$ $2H_2O$ 0.1 g; agar 9.0 g; pH 7.4. Sterilize by autoclaving (121°C, 20 min). Autoclave the desired Tween separately (121°C, 20 min). Cool the basal medium to 45–50°C, add the Tween to give a final concentration of 1.0%, shake until the Tween is completely dissolved and pour into plates. Inoculate the cultures as lines on the surface of the agar. Incubate for up to 7 to 14 days, inspecting daily. Look for an opaque halo around the growth [16].

Gelatin liquefaction

The gelatin hydrolysis tests for an organism's ability to break down the protein gelatin, which is derived from collagen. Gelatin causes the media to thicken, especially at cooler (below 28°C) temperatures. If the organism can release gelatinase enzymes, the gelatin is broken down or liquefied. The media is checked over a period of about a week after inoculation and incubation at room temperature, for gelatinase activity. The tube is placed on ice for a few minutes; and if the media fails to solidify, it is considered a positive test. The gelatinase reaction may be slow or incomplete.

The conventional methods require long periods of growth, long periods for development, or are difficult to interpret. Now, commonly use the trichloroacetic acid (TCA) enhancement to be more rapid and sensitive [17]. Prepare gelatin agar plates (per liter of deionized water): tryptic soy agar powder 40.0 g, gelatin 16.0 g. Make a single streak or spot of the microorganism from a stock culture onto a gelatin agar plate and/or casein agar plate and incubate at 30–35°C. Prepare a stock solution of 35% (W/V) TCA in deionized water. After incubation for 3 h (or 24 h for the casein hydrolysis test), flood the plate with the TCA solution. Look for occurrence of a clear zone around the growth within at least 4 min. With casein hydrolysis, clear zones may be visible without adding TCA, but the TCA enhances the visibility.

Coagulation and peptonization of milk

Milk coagulation and peptonization test the ability of actinobacteria to produce protease. Coagulation is that mild protein is preliminarily degraded into big pieces by organism. Further degradation is peptonization.

Prepare milk coagulation and peptonization medium (per liter of distilled water): skim milk powder 200 g; $CaCO_3$ 0.2 g. Dispense 3–5 ml portions into narrow tubes and sterilize by autoclaving (115°C, 15 min) or fractional sterilization for 2–3 times. Inoculate the tubes and observe in 5, 10, 20, 30 days, respectively. Milk solidification occurrence is the phenomenon of coagulation. Clots further hydrolyzed into liquid, is the phenomenon of peptonization. Peptonized exudates is translucent, typically begins after coagulation.

Starch hydrolysis

Starch hydrolysis tests the ability of an organism to produce certain exoenzymes, including a-amylase and oligo-1, 6-glucosidase, that hydrolyze starch. Starch molecules are too large to enter the bacterial cell, so some bacteria secrete exoenzymes to degrade starch into subunits that can then be utilized by the organism. Make a single spot of the test organisms on a plate of the agar (within 5 mm in diameter) and incubate.

Prepare a basal medium containing the following (per liter of distilled water): soluble starch 10 g; K_2PO_4 0.3 g; $MgCO_3$ 1 g; NaCl 0.5 g; KNO_3 1 g; agar 15 g; pH 7.2–7.4. Prepare Gram's iodine solution by grinding 1.0 g of iodine crystals together with 2.0 g of KI in a mortar (slowly add 300 ml of distilled water while grinding until the iodine is dissolved). After growth occurs, flood the plate with the iodine solution. Starch stains blue with iodine, so look for colorless areas around the microbial growth.

Cellulose hydrolysis

Cellulose hydrolysis tests the ability of an organism to produce cellulase.

Conventional method: Prepare a cellulose hydrolysis medium (per liter of distilled water): $MgSO_4$ 0.5 g; NaCl 0.5 g; K_2HPO_4 0.5 g; KNO_3 1 g; pH 7.2. A filter paper (5 × 0.8 cm) submerges in liquid medium. Sterilize by autoclaving (121°C, 20 min). Inoculate the cultures on the filter paper. Incubate for 1 month to observe whether filter paper is decomposed.

Congo red-polysaccharide method [18]:The interaction of the direct dye congo red with intact β-D-glucans provides the basis for a rapid and sensitive assay system for bacterial strains possessing β-(1 → 4),(1 → 3)-D-glucanohydrolase, β-(1 → 4)-D-glucanohydrolase, and β-(1 → 3)-D-glucanohydrolase activities. Prepare basal medium contain cellulose. Inoculate for 7 to 14 days. After growth occurs, flood the plate with the congo red (1 mg/ml) for 10–15 min. Wash with NaCl (1 mol/L) 2–3 times (15 min/time). Cellulose hydrolysis can produce transparent circle. Congo red can also be added directly to the medium.

Other method [19]: Prepare mineral agar culture media in which cellulose is to be provided as a sole carbon source (per liter of distilled water): KNO_3 0.5 g; K_2HPO_4 1.0 g; KCl 0.5 g; $MgSO_4$ $7H_2O$ 0.5 g; and agar, 15.0 g. Add 0.5 ml of a suitable trace metals solution. Autoclave, cool, and dispense into plates. Prepare a series of dilutions of the organism to be tested and spread 25–50 μl portions over the surface of the plates. Place a sterile disc of lens paper on the seeded surface of the plates and incubate for 3–7 days. Look for colonies that form visible holes in the paper. To increase visibility of the holes, stain the paper on the plates or after the paper is removed with 0.2% irgalan black in 2% acetic acid.

Nitrate reduction

Many organisms can respire anaerobically by using NO_3^- as a terminal electron acceptor for an electron transport system (nitrate respiration or dissimulator nitrate reduction). Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. The Griess reaction has more recently been employed to detect nitrite and nitrate as products of nitric oxide synthase in bacterial identification [20].

Preparation of recipes:

Nitrate reduction medium (per liter of distilled water): beef (meat) extract 3.0 g; KNO_3 1.0 g; peptone 5.0 g; pH 7.2–7.4.

Nitrite reduction medium (per liter of distilled water): beef (meat) extract 3.0 g; KNO_2 1.0 g; peptone 5.0 g; pH 7.2–7.4.

For either broth substrate, carefully weigh the ingredients and heat gently into solution. Dispense into test tubes and add inverted Durham tubes. Autoclave for 15 min at 121°C.

Reagent A (Griess A): Sulfanilic acid 0.8 g; acetic acid (5N) 100 ml.

Reagent B (Griess B): N, N-Dimethyl-α-naphthylamine 0.6 ml (replace alpha-naphthylamine); acetic acid (5N) 100 ml. (Fresh reagent has a very slight yellowish color.)

5N acetic acid is prepared by adding 287 ml of glacial acetic acid (17.4 N) to 713 ml of deionized water.

Zinc dust must be nitrate- and nitrite-free.

Protocol:

For either substrate, NO_3^- or NO_2^-, inoculate the medium with a heavy inoculum from well-isolated colonies of the test organism. Incubate at 28°C for 18–24 h, some actinobacteria need 7–14 days. When sufficient growth is observed in the tube, test the broth for reduction of the substrate.

For NO_3^- substrate: Observe for gas production in the Durham tube. Mix two drops each of reagents A and B in a small test tube. Add approximately 1 ml of the broth culture to the test tube and mix well. If the test organism has reduced the NO_3^- to NO_2^-, a red color will usually appear within 2 min, indicating the presence of NO_2^- in the tube. If no color change is seen within 2 min, there are several possible reasons. Either the organism (i) was unable to reduce NO_3^- at all, (ii) was capable of reducing NO_2^-, or (iii) reduced NO_3^- directly to molecular nitrogen. Zinc is a powerful reducing agent. If there is any NO_3^- remaining in the tube (option (i) above), a small amount of zinc dust will rapidly reduce it to NO_2^-. Therefore, the appearance of a red color after the addition of zinc dust to a colorless reaction tube indicates a negative reaction, i.e., the organism has failed to reduce NO_3^-. Zinc is added to the tube by dipping a wooden applicator stick in nitrate- and nitrite-free zinc powder, just enough to get the stick dirty, and then dropping it into the tube containing the culture broth and the reagents. If too much zinc is added, the color reaction may fade rapidly. If the broth remains colorless after the addition of zinc, the organism has also reduced the NO_2^-, intermediate product to N_2 gas or some other nitrogenous product. N_2 gas is usually visible in the Durham tube. In the absence of gas, the product is assumed to be other than N_2 gas.

Occasionally, a lighter pink color will appear after the addition of zinc dust because of partial reduction, i.e., some of the primary NO_3^- substrate remains in the tube. The original tube may be reincubated and retested the following day.

For NO_{2-} substrate: Observe for gas production on the surface and in the Durham tube. Mix two drops each of reagents A and B in a small test tube. Add approximately 1 ml of the broth culture to the test tube and mix well. If the test organism has reduced the NO_2^-, there will be no color change, indicating that all of the original NO_2^- is gone, i.e., reduced. Reduction is often confirmed by the presence of N_2 gas in the Durham tube or on the surface of the broth, but other nitrogenous products may be produced. Therefore, the absence of gas does not rule out reduction of NO_2^-. If a red color appears, it indicates the presence of NO_2^-, and therefore a negative reaction. Occasionally, a lighter pink color will appear because of partial reduction, i.e., some of the primary NO_2^- substrate remains in the tube. The original tube may be reincubated and retested the following day. There is no need to add zinc dust to this reaction.

Note:

1. Be sure to run a negative control, uninoculated broth, to illustrate that the remaining NO_2 will be reduced by zinc dust, producing a red color.

2. Because reduction of NO_3^- is assumed to be anaerobic, many published procedures warn that the medium needs to be anaerobic or deep enough to support an anaerobic process. However, later experiments have shown that the metabolism on the surface of the broth for most organisms that grow well in the broth will reduce enough dissolving oxygen for the reaction to take place.

3.7. Metabolic products

Thousands of characterization tests have been described in the microbiological identification. Those that follow are designed for detection of metabolic products and they are useful for physiological characterization beyond the more general features of an actinobacterial genus or species.

a. MR test (Methyl red test)

A type of fermentation called the mixed acid fermentation results in the formation of formic acid, acetic acid, lactic acid, succinic acid, ethanol, CO_2, and H_2 in a buffered medium. The combination of acids in the mixed acid fermentation usually lowers the pH of the culture below 4.2. The test is used mainly in the differentiation of enteric bacteria. The organism being tested must be capable of catabolizing glucose [21].

Prepare MR-VP medium containing the following (per liter of distilled water): peptone 7.0 g; K_2HPO_4 5.0 g; glucose, 5.0 g; pH 7.5. Dispense 2–3 ml portions into narrow tubes and sterilize by autoclaving. Inoculate the tubes lightly and incubate for 4 days at the optimum temperature for the organism. Add one drop of methyl red reagent (0.25 g methyl red dissolved in 100 ml of ethanol). Look for a red colour (MR positive). A weakly positive test is red orange and a yellow or orange color indicates a negative test.

b. V-P test (Voges-Proskauer test)

Some fermentative organism catabolizes glucose by the butanediol pathway, in which acetoin (acetylmethylcarbinol) occurs as an intermediate in the formation of 2, 3-butanediol. In the presence of KOH and O_2, the acetoin is oxidized to diacetyl, which in turn reacts with the guanidine group associated with arginine and other molecules contributed by peptone in the medium to form a pink- to red-colored product. The α-naphthol intensifies this color [21].

Prepare MR-VP medium containing the following (per liter of distilled water): peptone 7.0 g; K_2HPO_4 5.0 g; glucose, 5.0 g; pH 7.5. Make reagent A by dissolving 5.0 g of α-naphthol in 100 ml of absolute (100%) ethanol; the reagent must not be darker than straw color.

Prepare reagent B by dissolving 40.0 g of KOH in 100 ml of distilled water. Inoculate the tubes lightly and incubate for 2 days (routine test) and for 4 days (standard test) at the optimum temperature for the organism being tested.

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Add 0.6 ml of reagent A and agitate to aerate the medium. Add 0.2 ml of reagent B and again agitate the medium. Slant the tube to increase the aeration. Allow to stand for 15–60 min. Look for development of a strong cherry red color at the surface of the medium. A negative reaction shows no color or a faint pink to copper color.

c. Tryptophan decomposition (indole production)

Organisms that possess tryptophanase can carry out the following reaction: L-tryptophan → indole + pyruvic acid + NH_3. The indole can be detected by its ability to react with p-dimethylaminobenzaldehyde to form a quinoidal red-violet condensation compound [21].

Xylene extraction test is more sensitive than the conventional test. Grow the test organism in a suitable culture medium supplemented with 0.1–1.0% tryptophan. Avoid using media containing carbohydrates, nitrate or nitrite, as these may interfere with the test. Distribute in 2–3 ml portions and sterilize by autoclaving. When cool, inoculate with the organism to be tested and incubate for up to 3 days.

Prepare Ehrlich's reagent as follows: 1.0 g of p-dimethylaminobenzaldehyde, 95 ml of 95% ethanol and 20 ml of HCl.

Add 1 ml of xylene to the broth culture, shake vigorously, and allow the mixture to stand for about 2 min. Then add 0.5 ml Ehrlich's reagent slowly down the side of the tube so as to form a layer between the medium and the xylene. Do not shake the tube after addition of the Ehrlich's reagent. Look for development of a pink or red ring below the xylene layer.

d. Hydrogen sulphide production

Some anaerobic and facultatively anaerobic actinobacteria can produce abundant H_2S by the anaerobic reduction of $S_2O_3^{2-}$. The H_2S can be detected by its reaction with iron salts contained in the medium, which form a black precipitate of FeS. A different type of H_2S production is based on the ability of some organisms to form low levels of H_2S from sulfur-containing amino acids (cysteine, cystine, and/or methionine) by means of amino acid desulfurases. The gaseous H_2S so produced is detected by its reaction with lead acetate strips suspended above the surface of the medium.

Thiosulfate iron H_2S test [22]: Prepare peptone–iron agar medium (Tresner medium) as follows (per liter of distilled water): peptone 10.0 g; ferric ammonium citrate 0.5 g; agar 15 g. To achieve more satisfactory results, inoculums from actively growing cultures were used to streak the surface of the agar slants. After a short incubation period (15 to 20 h) at 28°C, the slants were observed. A pronounced bluish-black discoloration of the medium surrounding the colonies effected no change and indicated the production of hydrogen sulfide, whereas those organisms not producing H_2S in the medium emitted only faint tints of other colors.

Paper strip method [21]: Inoculate a suitable semisolid (0.2% agar) growth medium that contains a peptone or other source of sulfur amino acids. Suspend a strip of sterile, lead acetate-impregnated paper about a centimeter above the surface of the culture, fold the upper end over the lip of the tube, and hold it in place with the screw cap or cotton plug. During growth of the organisms, H_2S gas reacts with the lead acetate to form black PbS, beginning at the lower

part of the strip. Lead acetate strips can be prepared by soaking 5-cm strips of filter paper in a 5% aqueous solution of lead acetate, sterilizing them separately in tubes by autoclaving and drying them in an oven.

3.8. Relation to oxygen

Aerobes use O_2 as a terminal electron acceptor for an electron transport system, can tolerate a level of O_2 equivalent to or higher than that present in an air atmosphere (21% O_2), and have a strictly respiratory type of metabolism. Anaerobes are incapable of O_2-dependent growth and cannot grow in the presence of 21% O_2. Facultative anaerobes can grow both in the absence of O_2 and in the presence of 21% O_2. Microaerophiles respire with O_2 but cannot grow, or grow very poorly, under 21% O_2. They grow best at low O_2 levels; some require levels as low as 1%. Some microaerophiles can also respire anaerobically with electron acceptors other than O_2.

Semisolid agar method [23]: Autoclave a narrow culture tube that has been filled to 60% of its capacity with an appropriate culture medium containing 0.2% agar. After the medium has cooled to 45°C, add the inoculum, mix to distribute the organisms uniformly and then allow the agar to solidify. Alternatively, inoculate the medium by stabbing with an inoculating needle after the agar has gelled; this avoids the mixing that otherwise might add dissolved O_2 to the medium. Growth occurring only at the surface of the medium suggests that the organism is aerobic. However, a fermentable substrate should be present in the medium, because the organism might be a facultative anaerobe that not only respires with O_2 but also grows anaerobically by fermentation. Growth occurring only in the bottom region of the tube suggests that the organism is anaerobic. However, some extremely oxygen-intolerant anaerobes may not be able to grow even in the lowest region of the medium, because of the presence of small amounts of O_2 dissolved in the medium during the addition of the inoculum. Growth occurring throughout the tube suggests that the organism is a facultative anaerobe. It is important that no potential terminal electron acceptors other than O_2 should be present, as some aerobes can respire anaerobically. Growth occurring only in a disc several millimeters below the surface of the medium suggests that the organism is a microaerophile. Motile microaerophiles usually exhibit negative or positive aerotaxis, which results in their migration to a zone where the rate at which O_2 is diffusing to them matches the rate it is used by the organisms.

3.9. Susceptibility to antibiotics

Antibiotic sensitivity is the susceptibility of actinobacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic [Table 3] will be most successful in treating a bacterial infection in organism.

Testing for antibiotic sensitivity is often done by the Kirby-Bauer method [24]: wafers containing antibiotics are placed on an appropriate agar plate where actinobacteria have been placed, and the plate is left to incubate. If an antibiotic stops the actinobacteria from growing or kills the actinobacteria, there will be an area around the wafer where the bacteria have not grown enough to be visible. This is called a zone of inhibition. The size of this zone depends

on how effective the antibiotic is at stopping the growth of the bacterium. A stronger antibiotic will create a larger zone, because a lower concentration of the antibiotic is enough to stop growth.

Antibiotics	Concentration (per milliliter)
Amikacin	30 μg
Aureomycin	30 μg
Ciprofloxaci	10 μg
Chloramphenicol	30 μg
Erythromycin	15 and 30 μg
Gentamicin sulfate	10 μg
Kanamycin	15 μg
Netilmicin	10 μg
Novobiocin	5 and 30 μg
Oleandomycin	10 μg
Penicillin G	10 U
Polymyxin B	10 and 30 U
Streptomycin sulfate	10 and 25 μg
Terramycin	2.5 and 30 μg
Tetracycline	10 and30 μg
Tobramycin	10 μg
Vancomycin	10 μg

Table 3. Common antibiotics and the suitable concentration

3.10. Antibacterial activity detection

Actinobacteria are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents, and enzymes. Because of the excellent track record of actinobacteria in this regard, it is necessary to preliminarily screen antibacterial activity of isolated actinomycetes.

Common test strains: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Mycobacterium tuberculosis avium, Candida albicans, Aspergillus niger.* Also test strains can be selected according to necessity.

Cross streak method [25]: The actinomycetes isolates were inoculated at the centre of the sterile agar plates and the plates were incubated at 28°C for 5–7 days. After incubation, the nutrient broth (peptone 5 g; beef extract 3 g; NaCl 5 g; distilled water 1000 ml) cultures of test bacteria were streaked perpendicular to the growth of the actinomycete isolates. The plates were incubated at 37°C for 24–48 h and the extent of growth inhibition of the test bacteria was observed. The absence of growth or a less dense growth of test bacteria near the growth of actinomycete isolate was considered positive for the production and secretion of antibacterial metabolite.

Double-layer agar diffusion method [4]: Prepare double-layer agar plates; the lower is water agar (0.8–1% agar). Cool the basal medium (YIM 38 agar medium or LB agar medium) to 45–50°C, add the suspension of test strains, mix and spread on the water agar layer. Inoculate tested actinobacteria for 7 days at 28°C. Or pour fermentation broth of tested actinobacteria into sterilized steel rim or holes on medium and inoculate for 24–48 h at 37°C. Or inoculate the filter paper containing fermentation broth on the plates for 24-48 h at 37°C. Observe and record the diameter of inhibition zone. Results: no antibacterial activity (no zone of inhibition); weak antibacterial activity (diameter of zone, 6–15 mm), strong antibacterial activity (diameter of zone, >15 mm).

4. Commercial multitest system for identification of actinobacteria

In recent 40 years, with the development of microelectronics, computer, molecular biology, physics, chemistry, and subject crossing universality, a significant contribution of scientific and technological development to the clinical microbiology was the development of miniaturized identification systems based on classical method. Several systems are commercially available [Table 4], and new systems are being developed continually. These systems were mostly based on modifications of classical methods and were improved by the incorporation of highly sophisticated, computer-generated identification databases tailored for each system [26, 27]. Each manufacturer provides charts, tables, coding systems, and characterization profiles for use with the particular multi-test system being offered. These systems offer the advantages of miniaturization and are usually used in conjunction with a computerized system for identification of the organisms. As mentioned earlier, the use of these systems can increase standardization among various laboratories because of the high degree of quality control exercised over the media and reagents. Now actinobacteria is defined as a phylum of Gram-positive bacteria with high G+C content in their DNA. Although classical actinobacteria have the largest and most complex bacterial cells, some groups of actinobacteria possess the small and simple cell. For these simple (rod, cocci-shaped, without hyphae differentiated) actinobacteria, physiological and biochemical experiments are more important. Their physiological and biochemical tests can be carried by the automatic identification systems like the common bacteria.

Manufacturer	Test system	Designed for	Number of tests
bioMérieus	An-Ident	Anaerobes	21
	API 20E; API 20NE; API Rapid 20E	Enteric Gram-negative rods	20
	API 20 strep	*Enterococcoi, streptococci*	20
	API 50 CH	General use; based on carbohydrate catabolism	50
	API Campy	*Campylobacters*	10
	API Coryne	Coryneform rods	20
	API NH	*Haemophilus, Neisseria, Moraxella*	10
	API STAPH	*Staphylococci*	10
	API ZONE	Non-enteric Gram-negative rods	20
	GNI+	Aerobic and facultative anaerobic Gram-negative rods	28
	Vitek GPI	Gram-positive cocci; coryneform rods	30
	Vitek NGI	*Neisseria, Moraxella*, non-enteric Gram-negative rods	15
Biolog	Gen III	General use; based on reduction of tetrazolium salts when cells are oxidizing carbon sources	98
BD Diagnostic System	Crystal E/NF	Enteric and non-enteric Gram-negative rods	30
	Crystal Gram-Positive ID	Aerobic Gram-positive bacteria	29
	ID TRI Panel	Gram-negative and Gram-positive bacteria	30
	Oxi/Ferm Tub II	Fermentative, oxidase positive and non-fermentative Gram-negative rods	14
	Enterotube II	Enterobacteriaceae and other oxidase negative Gram-negative rods	15
Dade Behring Microscan	Rapid NEG ID3	Enterobacteriaceae and non-enteric Gram-negative rods	36
	Pos ID Type 2	*Streptococci, enterococci, staphylococci*	27
Remel	RapidID ONE	Enteric Gram-negative rods	19
	RapidID CB Plus	Coryneform rods	20
	RapidID NF Plus	Non-enteric Gram-negative rods	17
	RapidID NF	*Haemophilus, Neisseria, Moraxella*	13

Manufacturer	Test system	Designed for	Number of tests
	RapidID POS ID	*Streptococci* and *enterococci*	34
	RapidID STR	*Enterococci* and *streptococci*	14
Trek Diagnostic Systems	Sensititre AP80	Enterobacteriaceae and non-enteric Gram-negative rods	32
	Sensititre AP90	*Enterococci* and *streptococci*	32

The products information of manufacturer come from:

bioMérieux, Marcy l'Etoile, France (http:// www.biomerieux.com/servlet/srt/bio/portail/home);

Biolog, Hayward, CA. (http://www.biolog.com);

BD Diagnostic Systems, Franklin Lakes, NJ (http://www.bd.com);

Dade Behring, Inc., MicroScan Inc., West Sacramento, CA (now owned by Siemens Medical Solutions, Henkestraße 127, Erlangen 91052, Germany) (http://www.medical.siemens.com/webapp/wcs/stores/servlet/SMBridgeBq_catalogIdBe_-999B a_catTreeBe_100001Ba_langIdBe_-999Ba_storeIdBe_10001.htm);

Remel, Lenexa, Kansas (http://www.remel.com/clinical/ microbiology.aspx);

Trek Diagnostic Systems, Ltd., East Grinstead, West Sussex, UK (http://www.trekds.com).

Table 4. Some commercial multitest systems for prokaryote identification

4.1. API Numerical identification system

API (analytical profile index) is a classification of bacteria based on experiments, allowing fast identification. This system is developed for quick identification of clinically relevant bacteria. Because of this, only known bacteria can be identified. It was invented in the 1970s in the United States by Pierre Janin of Analytab Products, Inc. Presently, the API test system is manufactured by bioMérieux [28]. The API range introduced a standardized, miniaturized version of existing techniques, which up until then were complicated to perform and difficult to read.

API systems can determinate simultaneously more than 20 items of biochemical indicators. Choose appropriate API strip according to different bacterial groups. The API strip consists of more than 20 microtubes containing dehydrated substrates. The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software. Such as API 20NE is an identification system for non-fastidious, non-enteric Gram-negative rods [Figure 2].

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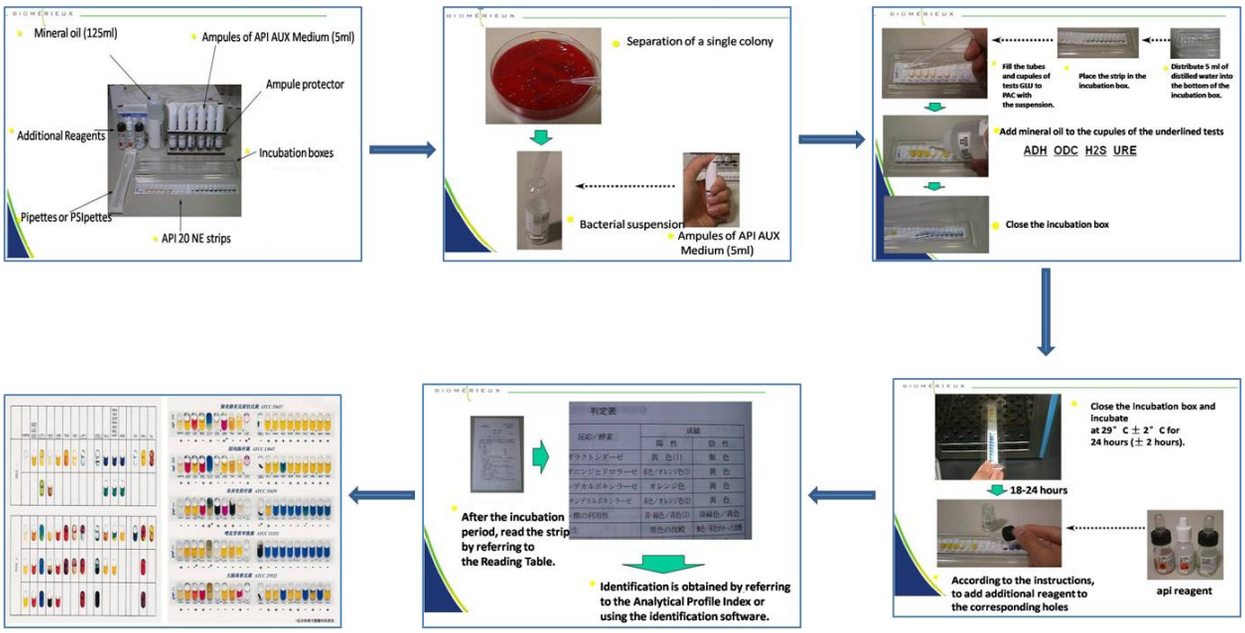

Figure 2. API 20NE operational flowchart

4.2. Biolog automatic bacterial identification system

The Biolog Microbial ID System (Biolog, Inc., Hayward, Calif.) can rapidly identify over 2,500 species of aerobic and anaerobic bacteria, yeasts, and fungi. These easy-to-use systems provide reference laboratory quality identifications. Biolog Systems do this without the labor-intensive requirements of conventional strips or panels [29, 30]. Biolog's latest generation redox chemistry enables testing and microbial identification of aerobic Gram-negative and Gram-positive bacteria in the same test panel. Gram stain and other pre-tests are no longer needed. A simple 1-min setup protocol and microbial samples are ready to be analyzed. Expanded GEN III database is designed to meet the needs of Biolog's broad customer base covering diverse disciplines of microbiology. All Biolog Microbial Identification Systems (manual, semi-automated, or fully automated) use the powerful new GENIII MicroPlate, allowing users to determine the most appropriate system to fit their current budget and level of throughput [Figure 3].

Figure 3. A common procedure of Biolog Microbial ID System [http://www.biolog.com]

It is important to realize that most such systems are designed for the identification of particular taxa and not for determining the physiological features of other taxa or new taxa. Indeed, a particular system may not even be applicable to other taxa. With these precautions in mind, multitest systems can provide useful information about the physiological characteristics of other organisms. For describing new taxa, the characterization systems that are used, as well as the inoculum age and size and the incubation temperature, must always be stated because reactions may not always agree with the results from classical characterization tests or with the results with other multitest systems.

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Author details

Qinyuan Li[1], Xiu Chen[1,2], Yi Jiang[1*] and Chenglin Jiang[1]

*Address all correspondence to: jiangyi@ynu.edu.cn

1 Yunnan Institute of Microbiology, School of Life Science, Yunnan University, Kunming, P. R. China

2 Institute of Microbial Pharmaceuticals, College of Life and Health Sciences, Northeastern University, Shenyang, P. R. China

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