SUBGROUP ON TAXONOMY OF
MICROAEROPHILIC ACTINOMYCETES

Report on Organization, Aims and Procedures

SUMMARY. A report of a working group of the Subcommittee on Taxonomy of the Actinomycetales of the International Committee on Nomenclature of Bacteria (ICNB). The group has been primarily concerned with recognition of type strains or other reference strains for the species of Actinomyces and with standardization of techniques for the study of the genus. Eleven species of Actinomyces or closely related organisms have been distributed and studied following techniques detailed in a "Manual of Procedures" which is included in the report.

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In 1961 as the result of an exchange of correspondence between Drs. John M. Slack, Arden Howell and Per Holm it was decided to collect and distribute cultures of Actinomyces to a number of interested workers in this field in an attempt to gather information about the reproducibility of results on morphological, physiological and antigenic characteristics of these cultures. Thirteen investigators participated in this study and the results were presented at a round-table discussion at the VIII International Congress of Microbiology in Montreal in August, 1962.

This was followed by the establishment in January, 1963 of a "Subgroup on Taxonomy of Microaerophilic Actinomycetes" as a division of the "Subcommittee on the Taxonomy of the Actinomycetes." This was done through the cooperative efforts of Dr. David Gottlieb. The membership of this subgroup is as follows:

* Subgroup on Taxonomy of Microaerophilic Actinomycetes, an authorized Subgroup of the Subcommittee on Taxonomy of the Actinomycetales of the International Committee on Nomenclature of Bacteria (ICNB), International Association of Microbiological Societies (IAMS).

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The initiation of actual laboratory work was preceded by a prolonged exchange of correspondence with the eventual decision that the initial aims of the committee should be:

- (1) the establishment and characterization of type cultures,
- (2) the standardization of techniques and criteria for the determination of the characteristics of first the genus and then the species, and
- (3) compilation of a glossary of terminology particularly in relation to morphological descriptions.

The question of standardizing techniques was argued pro and con with some favoring each laboratory continuing as usual and others for strict standardization. There was agreement that one of the major problems within this genus was the variability of reported results and that this variability arose primarily from the use of different media, varying inoculum size and varying oxygen concentrations. Therefore, it was decided that quite rigid techniques should be established even to the point of all participants using the same batch of media. Each laboratory would study cell and colonial morphology, perform tests for oxygen requirements and for certain physiological characteristics. These latter

tests include carbohydrate fermentation, starch hydrolysis, nitrate reduction, indol production, gelatin liquefaction, milk reactions, V-P, MR and catalase. In addition, at least two laboratories would investigate antigenicity, cell wall composition, pathogenicity, fermentation end products, DNA base ratios, and growth factors.

A manual of methods was prepared and its final edition is included at the end of this report.

Arrangements were then made with Dr. E. F. Lessel at the American Type Culture Collection (ATCC) for the distribution of cultures. He lyophilized single batches and distributed the following cultures to each member of the committee:

- Actinomyces israelii (Kruse) Lachner-Sandoval ATCC 10048 and 12102;
- A. naeslundii Thompson and Lovestedt ATCC 12104;
- A. bovis Hartz ATCC 13683;
- A. eriksonii Georg, Robertstad, Brinkman and Hicklin ATCC 15423;
- A. propionicus Buchanan and Pine ATCC 14157;
- A. odontolyticus (Rodriguez) Battey ATCC 17929;
- A. parabifidus (Weiss and Rettger) Pine and Georg (Lactobacillus parabifidus Weiss and Rettger) ATCC 17930;
- A. discofoliatus Grüter ATCC 19246;
- A. viscosus comb. nov. (Odontomyces viscosus Howell, Jordan, Georg and Pine) ATCC 15987;
- Rothia dentocariosa (Onishi) Georg and Brown (Actinomyces dentocariosus Onishi) ATCC 17931;
- Ramibacterium pleuriticum Prévot, Raynaud and Digeon ATCC 19301.

Results of the work of the Subgroup will be reported in the future.

MANUAL OF PROCEDURES

I. Media.

Baltimore Biological Laboratory furnished gratis the media which in each instance was from the same batch and included: Brain Heart Infusion Agar, Actinomyces Broth, Actinomyces Fermentation Broth, Indol-Nitrite Medium, M.R. - V.P. Medium, Litmus Milk, Yeast Extract, Soluble Starch and Gelatin.

II. Maintenance of Cultures.

- A. Frozen Stock Cultures: Remain viable for at least six months.

Medium: Actinomyces Broth + 0.3% agar. Tube in 5.0 ml amounts in 13 x 100 screw-capped tubes.

Procedure: Transfer cultures in Actinomyces Broth (subsequently written as A. Broth) at 3-4 day intervals until good growth is obtained. Inoculate the semi-solid medium by stabbing. Incubate at 37°C under 95% N₂ - 5% CO₂ (subsequently indicated as N₂-CO₂) for 4-7 days. Store at -21°C.

- B. Lyophilization: Remain viable for years.

Procedure: Inoculate two tubes of A. Broth. Incubate 4-7 days at 37°C under N₂-CO₂. Centrifuge and resuspend the growth in approximately 1.0 ml of sterile skim milk then lyophilize by standard techniques and finally check the tubes for vacuum.

III. Test for Oxygen Requirements.

Medium: Actinomyces Broth; Brain Heart Infusion agar (BHI) slants.

Procedure:

1. Inoculate 2 tubes of A. Broth with each test culture. Incubate 3-4 days at 37°C under N₂-CO₂.

2. Centrifuge the broth cultures. Pool the sediments and resuspend in sterile 0.85% NaCl. Adjust turbidity to an O.D. reading of 0.5 on a B & L Spectronic 20 or to match a MacFarland Standard No. 3.

3. Inoculate 8 BHI slants per culture using a capillary pipette. Place the tip of the pipette containing the well-mixed suspension at the bottom of the slant, force out one small drop, and draw the pipette in a single line from the base to the top of the slant. Incubate slants, in duplicate, under the different conditions as described below:

- a. For aerobic incubation, place two slants with the original cotton plugs in the 37°C incubator.
- b. Tubes to be incubated under aerobic + CO₂, anaerobic, and anaerobic + CO₂ conditions are handled as follows:

Clip off the cotton plug, push this short plug down into the tube to just above the slant and place a small pledget of absorbent cotton on top of the plug. Add reagents to the absorbent cotton as listed below and immediately close with a rubber stopper.

- Aerobic + CO₂ -- 5 drops of 10% Na₂CO₃ + 5 drops of 1 M KH₂PO₄
Anaerobic -- 5 drops of 10% KOH + 5 drops pyrogallol solution (pyrogallol solution = 100 grams pyrogallic acid + 150 ml distilled water)
Anaerobic + CO₂ -- 5 drops of 10% Na₂CO₃ + 5 drops of pyrogallol solution

Incubate all tubes at 37°C for 7 days and record results as follows:

- 3+ = the best growth under any one of the above conditions
2+ = medium growth in comparison to the best
1+ = slight growth in comparison to the best
0 = no growth

If the results on the duplicate tubes do not agree, the test should be repeated.

In testing an unknown culture, the O₂ requirement test should be done first so that all further tests can be done under conditions providing the best growth. Incubation under N₂-CO₂ has been specified in all the following descriptions of biochemical tests since this will be best for most Actinomyces. However, in all cases the tests should be incubated under the most favorable conditions for the culture being studied.

IV. Stains.

Gram stain - Hucker Modification, pg 16 "Manual of Microbiological Methods, 1957" Ziehl-Neelsen: p. 18 of the Manual.

V. Colonies.

Medium: Brain Heart Infusion agar (BHI): in order to facilitate microscopic observation of the colonies, pour the plates with no more than 10 ml of infusion agar.

Blood agar: Heart infusion agar + 5.0% defibrinated rabbit blood.

Microcolonies: Streak BHI plates and incubate at 37°C under N₂-CO₂. The colonies may be observed directly on the medium within the plate or a small section of the agar may be removed and placed on a glass slide. Observe the colonies at 18-24 hours and at 48 hours at 100X to 400X magnification. The lower magnification is useful for routine observations but higher magnification may be necessary for detailed study of the colony. For observation under oil immersion, place a sterile coverslip over the colony.

Macrocolonies: Streak both BHI and blood agar plates. Incubate at 37°C under N₂-CO₂ and observe at 7 and 14 days using a dissecting microscope.

VI. Catalase.

Prepare 3% H₂O₂ in distilled water from 30% H₂O₂ or use the commercial 3% solution. Store in the refrigerator. The catalase test can be done by adding the H₂O₂ solution to (1) colonies on BHI, (2) a loopful of the organism from a broth culture placed on a glass slide, (3) directly to the growth on a BHI slant. This last method gives the most reproducible results. If catalase is present, there will be an active production of bubbles. A positive control should always be included. All cultures should be held aerobically for 30 min. before testing.

VII. Carbohydrate Fermentation.

Medium:

Basal Medium: Actinomyces Fermentation Broth. Tube in 9.0 ml amounts in 16 x 150 mm tubes containing an inverted gas trap. Plug with cotton. Autoclave 15 min. at 121°C.

Complete Medium: All carbohydrates except the insoluble starch are added aseptically to the basal medium. For insoluble starch, 5 g is added to 1000 ml broth, tubed in 10 ml amounts, and autoclaved at 115°C for 10 min. Soluble starch, dextrin, glycogen and inulin - prepare a 5% solution in distilled water. Autoclave at 115°C for 10 min. Add 1.0 ml per tube of basal medium giving a 0.5% final concentration. All other carbohydrates: Prepare a 10% solution in distilled water. Filter sterilize. Add 1.0 ml to each tube of basal medium giving a final concentration of 1.0%.

Carbohydrates:

Monosaccharides: L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, alpha-methyl-D-glucoside, L-rhamnose, D-ribose, L-sorbose, and D-xylose.

Disaccharides: cellobiose, lactose, maltose, melibiose, sucrose, trehalose.

Trisaccharides: raffinose, melezitose.

Polysaccharides: dextrin, glycogen, inulin, insoluble potato starch, soluble potato starch.

Alcohols: adonitol, dulcitol, L-erythritol, glycerol, l-inositol, D-mannitol, salicin and D-sorbitol.

Miscellaneous: L(+) lactic acid (Na), sodium acetate, sodium pyruvate.

Procedure:

Use 3-4-day-old A. Broth cultures for inoculum. Mix growth with a pipette and add 0.1 ml to each tube of carbohydrate. For incubation, clip off cotton plug, push this short plug down into tube, and place a small pledget of absorbent cotton on top of the original plug. Add 5 drops pyrogallol solution + 5 drops 10% Na_2CO_3 . Include as controls a tube of uninoculated medium containing each carbohydrate and a tube of inoculated basal medium without carbohydrate.

Incubate at 37°C for 14 days observing frequently. Record as acid if the indicator has changed to yellow. If there is any question, test with a pH meter at the end of 14 days. A drop in pH of one unit from the pH of the uninoculated control is recorded as acid.

Note: The above method was used for comparative purposes by the Committee. However, a less cumbersome method which does not require sealing has been adequately tested and provides comparable results, it is as follows:

Medium: Thioglycollate broth without dextrose or indicator. To each 1000 ml add 2 g yeast extract without dextrose + 1 ml of 1% aqueous bromthymol blue. Tube in 9 ml amounts and autoclave 15 min. at 121°C. Add the carbohydrates as described above.

VIII. Starch Hydrolysis.

Medium: 1 liter Nutrient Agar + 5 g of soluble or insoluble starch. To prepare, suspend the starch in 40 ml cold water and add to the melted nutrient medium. Autoclave 15 min. at 121°C. Pour 15 ml into petri plates.

Procedure:

Using a capillary pipette transfer 1 drop of A. Broth culture to one side of the plate and pull this drop across the surface of the medium. Inoculate the other half of the plate with a second culture. Inoculate in triplicate plates of both soluble and insoluble starch.

Incubate in $\text{N}_2\text{-CO}_2$ at 37°C. Test one set of plates at 3 days, another set at 7 days, and the last set at 14 days.

To test, flood the plate with 2-4 ml of Gram's iodine. No hydrolysis: entire plate turns blue. Complete hydrolysis: colorless area around the colony or along the streak. Partial hydrolysis: a pink area around the growth.

IX. Gelatin Hydrolysis.

Medium: 1000 ml A. Broth + 15 g agar + 4 g gelatin (soak gelatin in 40 ml of cold water and mix with the melted agar). Autoclave 15 min. at 121°C. Pour 15 ml into petri plates.

Procedure:

Using a capillary pipette inoculate duplicate plates as described above and incubate at 37°C in N₂-CO₂. Test one set at 7 and the other at 14 days.

To test, flood the plate with 2-4 ml of Bichloride of Mercury (15 g HgCl₂ + 20 ml conc. HCl + 100 ml distilled water). The medium will turn cloudy. Where hydrolysis has occurred there will be a clear zone around the colony or streak.

X. Indol-Nitrite.

Medium: Indol-Nitrite Broth - 5 ml per tube. Autoclave 15 min. at 121°C. If tubes are to be sealed individually they should be cotton plugged. Metal-capped tubes may be used if incubation is to be in an anaerobic jar.

Procedure:

Inoculate 4 tubes with 0.05 ml each of growth from A. Broth. Incubate at 37°C in N₂-CO₂. Test at 3 and 7 days using one tube for indol and one for nitrite.

Indol: Add 1 ml of xylene to the culture, shake, and allow to stand 3-5 min. Add 4-5 drops of Ehrlich's Reagent (1 g para-dimethyl amino benzaldehyde + 95 ml ethyl alcohol + 20 ml conc. HCl). A red ring developing at the broth-xylene interface is positive. Include positive and negative controls.

Nitrite:

Reagents: (1) 8 g of sulfanilic acid + 1000 ml of 5 N acetic acid. (2) 5 g of alpha-naphthylamine + 1000 ml of 5 N acetic acid.

Test: Add 5 drops of Reagent 1 and then 5 drops of Reagent 2. The development of a red color shows that nitrite is present. If no color develops, the organism did not reduce nitrate or reduced both nitrate and nitrite. To distinguish between these reactions, add a small amount of zinc powder to the tube. If a red color develops this shows that nitrate was still present in the tube and was not reduced by the bacteria. If the tube remains colorless after the addition of zinc, this shows that both nitrate and nitrite have been reduced. Include both known positive and negative controls.

XI. Methyl Red, Voges-Proskauer.

Medium: MR-VP Medium + 5 g yeast extract per liter, 5 ml per tube. Plug or cap the tubes as described under Indol.

Procedure: Inoculate four tubes with 0.05 ml each of the growth from an A. Broth culture. Incubate at 37°C in N_2 -CO₂. Test at 3 and 7 days.

Methyl Red:

Reagent: Dissolve 0.1 g methyl red in 300 ml of 95% ethyl alcohol and dilute to 500 ml with distilled water.

Test: Add 5 drops of MR Indicator and shake gently. If the indicator remains red the test is positive.

Voges-Proskauer:

Reagent: (1) 5 g alpha-naphthol + 100 ml absolute ethyl alcohol. (2) 40 g potassium hydroxide + 0.3 g creatine + 100 ml distilled water.

Test: To 1.0 ml of culture add 0.6 ml of alpha-naphthol reagent and 0.2 ml of the KOH Reagent. Shake the tube and at the same time warm gently in the Bunsen flame. Then remove from the flame and shake vigorously for 30 seconds. The development of a crimson to ruby color in 2-5 min. is positive.

Nitrate, indol, M.R. and V.P. tests may be incubated by sealing each tube with the appropriate reagents as described under oxygen requirements if jars for incubation are not available.

XII. Hydrogen Sulfide.

Medium: Brain Heart Infusion agar slants with cotton plugs.

Procedure: Inoculate the slant with one drop of a well-mixed A. Broth culture using a capillary pipette. Insert a lead acetate paper strip into the top of the tube. Incubate at 37°C under N_2 -CO₂. Observe for blackening of the lead acetate paper after 7 and 14 days. Do not use rubber stoppers as they may cause the strips to blacken. (Lead acetate strips may be prepared by soaking strips of filter paper in a saturated solution of lead acetate. Dry and sterilize in the hot air oven.)

XIII. Milk.

Medium: Litmus milk or skim milk + 5 g yeast extract per liter.

Procedure: Inoculate with 0.1 ml of well-mixed A. Broth culture. Seal with a pyrogallol-carbonate seal or incubate

at 37° C in N₂-CO₂. Observe for coagulation after 3 and 7 days. It is difficult to determine acid production and other reactions in milk even when using the litmus milk, thus record only the presence or absence of clotting.

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