Antibiotic producing organisms isolated from the White River Canal, Indianapolis, Indiana

Fred C. Mindach

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The Butler University Botanical Studies journal was published by the Botany Department of Butler University, Indianapolis, Indiana, from 1929 to 1964. The scientific journal featured original papers primarily on plant ecology, taxonomy, and microbiology.

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Butler University
Botanical Studies
(1929-1964)

Edited by

Ray C. Friesner
The *Butler University Botanical Studies* journal was published by the Botany Department of Butler University, Indianapolis, Indiana, from 1929 to 1964. The scientific journal featured original papers primarily on plant ecology, taxonomy, and microbiology. The papers contain valuable historical studies, especially floristic surveys that document Indiana’s vegetation in past decades. Authors were Butler faculty, current and former master’s degree students and undergraduates, and other Indiana botanists. The journal was started by Stanley Cain, noted conservation biologist, and edited through most of its years of production by Ray C. Friesner, Butler’s first botanist and founder of the department in 1919. The journal was distributed to learned societies and libraries through exchange.

During the years of the journal’s publication, the Butler University Botany Department had an active program of research and student training. 201 bachelor’s degrees and 75 master’s degrees in Botany were conferred during this period. Thirty-five of these graduates went on to earn doctorates at other institutions.

The Botany Department attracted many notable faculty members and students. Distinguished faculty, in addition to Cain and Friesner, included John E. Potzger, a forest ecologist and palynologist, Willard Nelson Clute, co-founder of the American Fern Society, Marion T. Hall, former director of the Morton Arboretum, C. Mervin Palmer, Rex Webster, and John Pelton. Some of the former undergraduate and master’s students who made active contributions to the fields of botany and ecology include Dwight. W. Billings, Fay Kenoyer Daily, William A. Daily, Rexford Daubenmire, Francis Hueber, Frank McCormick, Scott McCoy, Robert Petty, Potzger, Helene Starcs, and Theodore Sperry. Cain, Daubenmire, Potzger, and Billings served as Presidents of the Ecological Society of America.

Requests for use of materials, especially figures and tables for use in ecology text books, from the *Butler University Botanical Studies* continue to be granted. For more information, visit www.butler.edu/herbarium.
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**agonistic effects of micro-**

**Sci. 58:89-114. 1944.**

**ANTIBIOTIC PRODUCING ORGANISMS ISOLAT-**

**ED FROM THE WHITE RIVER CANAL, INDI-**

**ANAPOLIS, INDIANA**

By Fred C. Mindach

Although a number of antibiotic producing organisms have been isolated from soil, especially in recent years; little work has been reported on either microbial antagonisms or on the isolation of antibiotic producing organisms from water. A review of the literature indicates that no specific antibiotic substance has been reported for microorganisms isolated from either fresh or salt water.

Frankland (3) was the first to establish that *Eberthella typhosa* may survive in sterilized polluted water or in deep-well water for 20 to 51 days although it dies out in 9 to 13 days in unsterile surface water. Jordan, Russell, and Zeit (8) found that the typhoid organism was able to survive in sterilized tap water for 15 to 25 days, as against 4 to 7 days in fresh water; the bacteria died off even more rapidly in raw river or canal water, the survival time being reduced to 1 to 4 days. The degree of survival of typhoid organism in water was found to be in inverse ratio to the degree of contamination of the water, the saprophytic bacteria in the water apparently being responsible for the destruction of the pathogen. These conclusions were later confirmed.

Vacek (12) showed that among the factors responsible for the disappearance of *Eberthella typhosa* in water, the presence of certain water bacteria was found to be of special importance. Rochaix and Vieux (10) demonstrated that when an achromogenic strain of *Pseudomonas aeruginosa* was present in drinking water, it was not accompanied by any other bacteria. Media inoculated with this organism and *Escherichia coli* gave, after 13 days' incubation, only cultures of the former. That the two organisms could coexist, however, was shown by inoculation into sterilized water. Only the actual develop-

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*A portion of a thesis submitted in partial fulfillment of the requirements for the Master of Science degree in the Division of Graduate Instruction, Butler University.*
ment of the antagonist led to the repression of the fecal organism. Studies made in Malta by Gilmour (5) and Horrocks (6) brought out the fact that *Brucella melitensis* survived in sterile tap water 42 days and in unsterile tap water only 7 days.

Hutchinson, Weaver, and Scherago (7) while making a study of microorganisms antagonistic to *E. coli*, isolated organisms from 5 of 44 samples of well water, 1 of 12 samples of spring water, and 6 of 16 samples of surface water. The antagonists included 3 strains of *Pseudomonas*, 1 each of *Sarcina*, *Micrococcus*, *Flavobacterium*, and yeast, 2 actinomycetes, and 3 unidentified nonspore-forming gram-negative rods. No species identifications were cited in their work.

According to Waksman and Hotchkiss (14) and Zobell (15) sea water, also, appears to have a bactericidal effect upon organisms added to it. However, almost fifty years before, De Gioux (4) observed that pathogens rapidly perish in raw sea water, although they may survive almost indefinitely in heat-treated sea water. Water from the Black Sea was found by Krassilnikov (9) to be germicidal for terrestrial bacteria until it is boiled. He confirmed the observations of Beard and Meadowcraft (1) and Zobell (15) that the bactericidal potency of sea water was decreased but not destroyed by passing it through Berkefeld, Chamberland, Coors, or similar filters. Rosenfeld and Zobell (11) concluded that, lacking the properties of bacteriophage, the bactericidal property of sea water is attributed primarily to its content of antibiotic substance produced by microorganisms. Credence is lent to this view by the observation that the bactericidal principle occurs in greatest concentration in samples of sea water recently collected from zones of maximum bacterial population. Of the 58 microorganisms isolated by Rosenfeld and Zobell from sea water, 9 showed antagonism toward fresh water or terrestrial organisms. Broken down into genera, the results are as follows: Bacillus, 4 of 9; *Micrococcus*, 3 of 6; *Actinomyces*, 1 of 2; *Serratia*, 1 of 1; *Pseudomonas*, 0 of 19; *Vibrio*, 0 of 11; *Flavobacterium*, 0 of 5; *Achromobacter*, 0 of 4; *Sarcina*, 0 of 1.

Waksman (13) emphasizes the fact that there are marked differences in the nature of the microbial population of waters and soils because of the physical and chemical differences in the composition of the two substrates. However, he continues by saying that some of the underlying principles, example, marked differences in populations of soil and water substrates. Whereas, microorganisms perish at a very rapid rate, those populations of soil and water are nearly static, since the rates of growth of microorganisms except under very special conditions are very slow. This is evident not only in the chemical nature of organic matter or in the chemical nature of microorganisms but also in the microbiological populations of soil and water substrates. This work began as an attempt to isolate microorganisms from surface waters, to determine the number of antibiotic producing microorganisms, and to study the isolation of microorganisms from marine waters. Microorganisms, tentatively identified as *Flavobacterium rigense*, showed antagonism to other organisms in the initial tests.

As mentioned earlier, Scherago (7) reported the isolation of microorganisms which showed antagonism to *E. coli* and other bacteria. Rosenfeld and Zobell (11) made to species identification of their isolated strains of *Flavobacterium rigense*, and will be dealt with only in a general manner. Two of these organisms, named *Flavobacterium rigense*, showed little antagonism to other bacteria, and will be dealt with only in a general manner. As mentioned earlier, Scherago (7) reported the isolation of microorganisms which showed antagonism to *E. coli* and other bacteria. Rosenfeld and Zobell (11) made to species identification of their isolated strains of *Flavobacterium rigense*, and will be dealt with only in a general manner.
of the underlying principles apply to all substrates. There are, for example, marked differences in the nature and abundance of the populations of soil and water and those of milk, sewage, and foodstuffs. Whereas, microorganisms multiply in the latter substrates at a very rapid rate, those in the soil and in water basins are more nearly static, since the rate of their multiplication is much slower except under very special conditions, such as the addition of fresh, undecomposed plant and animal residues or a change in the environment or in the chemical nature of the substrate. Furthermore, the microbiological populations of soils, composts, and water basins are also influenced markedly by seasonal and temperature changes.

This work began as an attempt (1) to isolate antibiotic producing organisms from surface (river) water, and (2) if such isolations were possible, to determine the relationship between the maximum number of antibiotic producing organisms isolated and the bacterial population during the various seasons of the year.

Although circumstances necessitated altering the original problem, it is significant to note that preliminary investigation resulted in the isolation of microorganisms showing antagonism to other organisms. Two of these organisms, tentatively identified as Achromobacter iophagus, showed little or no antagonism toward test organisms and will be dealt with only briefly in this paper. However, two other microorganisms, tentatively identified as Bacillus megatherium and Flavobacterium rigense, showed appreciable antibiosis toward known organisms in the initial tests.

As mentioned earlier in this paper, Hutchinson, Weaver, and Scherago (7) reported the isolation of a strain of Flavobacterium showing antagonism to E. coli. However, no specific reference was made to species identification or the degree of antagonism produced. Rosenfeld and Zobell (11) isolated four species of Bacillus which showed antagonism to other organisms but, being salt water species, they are not directly comparable to Bacillus megatherium. Since none of their isolated strains of Flavobacterium showed antagonism to other organisms, no species identifications were mentioned in their report. To this writer's knowledge, no strain of Bacillus megatherium or Flavobacterium rigense, showing antagonism to other microorganisms, have been isolated before from fresh or salt water.
Due to the fact that preliminary procedures proved too time-consuming, it was decided to limit the present study to a more thorough investigation of the antagonistic properties of the isolated strains of \textit{Bacillus megatherium} and \textit{Flavobacterium rigens}. An attempt was made to determine (1) the production of antibiotic substances by the two organisms, and (2) the culture and incubation conditions under which maximum inhibitions were produced.

**PROCEDURE**

The water samples used for this work were collected from the White River Canal near the foot bridge on the Butler University campus. The canal water has its source from White River at the Broad Ripple headgates in the northeast section of Indianapolis. The original plan called for the collection of samples once a week for a period of one year.

On October 27, November 4, and November 8, 1947 water samples were collected in sterile, 180-ml aluminum-capped bottles. Special attention was given to sampling technique in order to make reasonably sure that any organisms isolated from laboratory cultures would be from the water and not from the air or other sources. Agar dilution plates were made from the water samples immediately upon arrival at the laboratory. The platings were completed within an hour after the samples were collected.

One series of agar dilution plates was made to determine bacterial counts. Dilutions ranging from 1 ml up to and including 1:10,000 were made, plated in nutrient agar, and incubated at 37.5° C. for 24 hours.

Two other series were made with dilutions ranging from 1 ml up to and including 1:10,000,000. These dilutions were plated with nutrient agar and a modified Czapek-Dox medium which will be referred to as C-D 1 medium in this paper. The pH of the former (organic) medium was reduced to 4.5, while no pH adjustment was made on the latter (synthetic-organic) medium. The pH of this medium was approximately 7.0. By using these media it was hoped that growth of fungi and true Actinomyces would be enhanced, while other organisms might be inhibited.

*Formulae for these media*

\begin{align*}
\text{Bacto-Beef Extract} & \\
\text{Bacto-Peptone} & \\
\text{Bacto-Agar} & \\
\text{Distilled Water} & \\
\text{Bacto-Dextrose} & \\
\text{Bacto-Peptone} & \text{KH}_2\text{PO}_4 & \\
\text{MgSO}_4 \cdot 7 \text{H}_2\text{O} & \text{Fe SO}_4 \cdot 7 \text{H}_2\text{O} & \\
\text{Bacto-Agar} & \text{Distilled Water} & \\
\text{Bacto-Dextrose} & \\
\text{Bacto-Peptone} & \text{NaNO}_3 & \\
\text{KH}_2\text{PO}_4 & \\
\text{Bacto-Agar} & \\
\text{Distilled Water} & \\
\text{Bacto-Dextrose} & \\
\text{Bacto-Peptone} & \text{KH}_2\text{PO}_4 & \\
\text{MgSO}_4 \cdot 7 \text{H}_2\text{O} & \text{Fe SO}_4 \cdot 7 \text{H}_2\text{O} & \\
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\text{Bacto-Peptone} & \text{KH}_2\text{PO}_4 & \\
\text{MgSO}_4 \cdot 7 \text{H}_2\text{O} & \text{Fe SO}_4 \cdot 7 \text{H}_2\text{O} & \\
\text{Bacto-Agar} & \text{Distilled Water} & \\
\end{align*}*

*Taken from the American Type Culture Collection.*
Formulae for these media are as follows.

**NUTRIENT AGAR**

- Bacto-Beef Extract: 3 gm
- Bacto-Peptone: 5 gm
- Bacto-Agar: 15 gm
- Distilled Water: 1 liter

**C-D 1 AGAR**

- Bacto-Dextrose: 20 gm
- Bacto-Peptone: 5 gm
- KH₂PO₄: 0.5 gm
- MgSO₄ • 7 H₂O: 0.25 gm
- Fe SO₄ • 7 H₂O: 0.01 gm
- Baclo-Agar: 15 gm
- Distilled Water: 1 liter

The bacterial counts for the three agar dilution series were as follows: October 27, 4,600 colonies per ml; November 4, 4,200 colonies per ml; and November 8, 2,600 colonies per ml. Counts for the canal water* showed a maximum bacterial count of 650,000 colonies, a minimum of 200 colonies, and an average of 7,000 colonies per 0.1 ml. Temperatures of the samples of water collected were 64°F, 56°F, and 46°F, respectively. The average yearly temperature was 55°F.

The agar dilution plates with nutrient agar (pH 4.5) and the C-D 1 agar were incubated at room temperature (approximately 25°C.) until good growth occurred on the plates, usually 24-96 hours. Only well isolated colonies were sub-cultured to agar plates containing the same medium as the plates from which the colonies were isolated. Once again a well isolated colony was selected and transferred to test tube agar slants of nutrient agar (pH 6.8) and also to slants of C-D 2 (synthetic) agar medium, another modification of the Czapek-Dox medium.

**C-D 2 Agar**

- Bacto-Dextrose: 40 gm
- NaNO₃: 2 gm
- KH₂PO₄: 1 gm

* Taken from the Annual Laboratory Report of the Indianapolis Water Company.
MgSO₄ · 7 H₂O ............................ 0.5 gm
Bacto-Agar ..................................15 gm
Distilled Water ............................ 1 liter

The purpose of sub-culturing these organisms on nutrient agar (pH 6.8) and on C-D 2 agar was to prove their ability to grow on these media before subsequent culture work was attempted.

From the three series of agar plate dilutions, sixty-seven organisms were isolated. Macroscopically, all but four appeared to be bacteria; the others appeared to be fungi. Due to an unavoidable lapse of time, twenty-two of these cultures were lost.

A single streak of each of the forty-five cultures remaining was made on pour-plates of nutrient agar (pH 6.8) and also on C-D 2 agar. The agar plates were incubated at room temperature until good growth of the unknown organisms appeared. Then approximately one inch streaks of Micrococcus catarrhalis, E. coli, Staphylococcus aureus, and Penicillium chrysogenum were made at right angles to and just in contact with the growth of the unknown organisms. The plates were incubated again at room temperature for 96 hours and observed at 24 hour intervals for evidence of inhibition.

In preliminary tests four of the forty-five unknown organisms tested showed inhibition to one or more of the known test organisms. These included unknowns Nos. 10 and 19, isolated from the water collected on October 27, 1947, and unknowns Nos. 28 and 41, isolated from the water collected on November 4, 1947. Inhibition zones ranged from 0 to 25 mm.

Tryptose-phosphate broth and agar media (pH 7.3) and nutrient broth and agar media (pH 6.8) were selected as the culture media for further work.

### TRYPTOSE-PHOSPHATE MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Tryptose</td>
<td>20 gm</td>
</tr>
<tr>
<td>Bacto-Dextrose</td>
<td>2 gm</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 gm</td>
</tr>
<tr>
<td>Disodium Phosphate</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1 liter</td>
</tr>
<tr>
<td>15% Bacto-Agar (added for broth enrichment)</td>
<td></td>
</tr>
<tr>
<td>2.0% Bacto-Agar (added for solid medium)</td>
<td></td>
</tr>
</tbody>
</table>

In the first series, try flasks were inoculated with several 250-ml Erlenmeyer flasks using agar medium. The dilutions of culture broth plus 18 ml of medium in order to insu excessive shrinkage of medium in order to insu
the organisms on nutrient agar as to prove their ability to grow on these media was attempted. By plate dilutions, sixty-seven organisms, all but four appeared to be bacteria. Due to an unavoidable lapse in the laboratory, the forty-five cultures remaining was lost.

Eighty to ninety ml of the broth media were transferred to each of several 250-ml Erlenmeyer flasks. These flasks were cotton-stoppered and sterilized at 15 lbs. steam pressure for 15 minutes. This volume of medium allowed a large surface for growth of the organism and also allowed for greater concentration of any antibiotic substance that might be produced.

Two hundred and fifty ml of the 2.0% agar media were transferred into separate 500-ml Erlenmeyer flasks, which were also cotton-stoppered and sterilized at 15 lbs. steam pressure for 15 minutes.

When the agar dilution plates were made, the melted agar, cooled to approximately 50 to 55° C., was pipetted in amounts sufficient to satisfy any dilutions made. Later, volumes of solid media necessary for the various dilutions were placed in separate culture tubes before sterilizing as above. Although this procedure seemed less accurate, it was found, after several dilution series had been made, that it was impossible to make many dilutions by pipetting before the cooled agar began to solidify. This either caused lumpy plates or necessitated repetition of portions of some series. In the first series, tryptose-phosphate broth and nutrient broth flasks were inoculated with the four unknown organisms and incubated at 37.5° C. These cultures were allowed to incubate until good growth was evident or until such time as it was thought necessary to occur. Subsequent series at different incubation temperatures were treated in the same manner.

The first tests were usually made after the cultures had incubated from 3-5 days. Agar dilution plates were made from the culture flasks using agar medium corresponding to the original broth culture medium. All dilution plates were prepared to contain a final volume of 20 ml of medium in order to insure sufficient growing medium and to avoid excessive shrinkage of medium during incubation.

<table>
<thead>
<tr>
<th>NUTRIENT MEDIUM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Beef Extract</td>
<td>3 gm</td>
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</tr>
<tr>
<td>Distilled Water</td>
<td>1 liter</td>
</tr>
<tr>
<td>15% Bacto-Agar (added for broth enrichment)</td>
<td></td>
</tr>
<tr>
<td>20% Bacto-Agar (added for solid medium)</td>
<td></td>
</tr>
</tbody>
</table>

Eighty to ninety ml of the broth media were transferred to each of several 250-ml Erlenmeyer flasks. These flasks were cotton-stoppered and sterilized at 15 lbs. steam pressure for 15 minutes. This volume of medium allowed a large surface for growth of the organism and also allowed for greater concentration of any antibiotic substance that might be produced.

Two hundred and fifty ml of the 2.0% agar media were transferred into separate 500-ml Erlenmeyer flasks, which were also cotton-stoppered and sterilized at 15 lbs. steam pressure for 15 minutes.

When the agar dilution plates were made, the melted agar, cooled to approximately 50 to 55° C., was pipetted in amounts sufficient to satisfy any dilutions made. Later, volumes of solid media necessary for the various dilutions were placed in separate culture tubes before sterilizing as above. Although this procedure seemed less accurate, it was found, after several dilution series had been made, that it was impossible to make many dilutions by pipetting before the cooled agar began to solidify. This either caused lumpy plates or necessitated repetition of portions of some series.

In the first series, tryptose-phosphate broth and nutrient broth flasks were inoculated with the four unknown organisms and incubated at 37.5° C. These cultures were allowed to incubate until good growth was evident or until such time as it was thought necessary to occur. Subsequent series at different incubation temperatures were treated in the same manner.

The first tests were usually made after the cultures had incubated from 3-5 days. Agar dilution plates were made from the culture flasks using agar medium corresponding to the original broth culture medium. All dilution plates were prepared to contain a final volume of 20 ml of medium in order to insure sufficient growing medium and to avoid excessive shrinkage of medium during incubation.
After dilutions had been made, and the agar had hardened thoroughly, the bottoms of the petri plates were sectioned and numbered with a china-marking crayon. Then, the plates were placed upright, and loop inoculations of aqueous suspensions of known test organisms were made on the surface of the culture medium in corresponding numbered sections of all plates. Control plates were made for each series. Fresh aqueous suspensions of the test organisms were made for each dilution series if more than a week's time elapsed between series. However, experiments proved that two-week-old suspensions grew readily on control plates. These suspensions were placed in the refrigerator when not in use.

After inoculation, the agar dilution plates were placed in the incubator in an upright position to avoid the spreading of inoculations into adjacent areas. In this manner, multiple cross-sectioning of the plates allowed for as many as eight or more inoculations of test organisms on the same plate. After 24 hours' incubation at 37.5°C, the plates were removed from the incubator and observed. Growth or lack of growth of the test organisms in the various dilutions was recorded. Only complete inhibition of the test organisms was recorded as lack of growth, and no attempt was made to measure partial or retarded growth.

After agar dilution plates had been made, the culture broth flasks were incubated for an additional period of time, approximately one week, and then the dilution series were repeated. Several difficulties were encountered in the initial dilution series, and a number of trials were made before the final procedure was adopted.

The procedure finally adopted was that of centrifuging: culture broth was transferred to large, sterile culture tubes and centrifuged at 1800 rpm for 15 minutes. The supernatant liquid was carefully transferred to other sterile culture tubes and centrifuged again at 1800 rpm for 15 minutes. This supernatant liquid was used for agar dilution plates. This procedure did not eliminate all organisms from the culture broth in some cases, but their growth on agar dilution plates was significant only in low dilutions. Condensation was overcome by removing the glass tops after the agar had been poured and allowed to solidify, and replacing them with sterile clay tops which were porous enough to absorb the condensate due to incubation.

In the subsequent section, special consideration was given to the conditions for the production of known organisms. In this way, known organisms were added for the production of the product. Where C. pneumoniae was allowed to inoculate the additional 24 hours for growth of the organisms, with the exception of the number of dilution series plates after 24 hours' incubation, no agar dilution plates were made.

The strains of the known organisms used for the identification are cited, and discussed below.

**Organism:** *Bacillus subtilis*.

**Shape:** Rods.

**Size:** 1 to 2 μm.

**Grouping:** Single, pairs, or short chains.

**Motility and Cilia:** Motile, flagella.

**Gram stain:** Negative.

**Spores:** Paraterminal, 0.8 to 1.0 μm.

**Cell contents:** Gelatin stab.

**Gelatin stab:** Agar plate.

**Nutrient broth:**
In the subsequent series of broth cultures and agar plate dilutions, special consideration was given to the variation of incubation temperatures. In this way, optimum growing temperatures for the unknown organisms were ascertained. Also, the optimum growing conditions for the production of antibiotic substances by the organisms were determined. When agar dilution plates were incubated at 20°C, they were allowed to incubate for 48 hours. The primary purpose of this additional 24 hour incubation period was to insure sufficient time for growth of the known test organisms. However, all test organisms, with the exception of Saccharomyces pastorianus in a number of dilution series, showed very satisfactory growth on control plates after 24 hours' incubation. Although broth cultures were grown at room temperature (approximately 25°C) for some series, no agar dilution plates were incubated at this temperature.

The strains of the known test organisms, acquired from the Eli Lilly Company and Butler University laboratories were: Micrococcus catarrhalis (Lilly), E. coli (Lilly), Staphylococcus aureus (Lilly), Monilia albicans (Lilly), Saccharomyces pastorianus (Lilly), Pseudomonas aeruginosa (Butler) Serratia marcescens (Butler), Sarcina lutea (Butler).

IDENTIFICATION OF UNKNOWN ANTIBIOTIC-PRODUCING ORGANISMS

Bergey's Manual of Determinative Bacteriology (6th edition) was used for the identification. Any variations from Bergey's identification are cited, and discussion is given.

UNKNOWN NO. 10

Organism: Bacillus megatherium. Bergey, pp. 714-715

Shape: rods
Size: 1.5 microns by 3.0 to 5.0 microns
Grouping: singly, pairs, short chains
Motility and Cilia: Motile, flagellation not observed (Bergey: peritrichous flagella)
Gram stain: Gram positive
Spores: paracentral to paraterminal, .8 to 1.0 micron by 1.0 to 1.5 microns, cylindrical, not swollen
Cell contents: stain unevenly (vacuolated
Gelatin stab: slow liquefaction, crateriform to infundibuliform
Agar plate: creamy white, moist, with pellucid dots
Nutrient broth: heavy uniform turbidity
Purple milk..............................................peptonization
Potato..............................................abundant, slimy, creamy white changing to brown
Indole...............................................not produced (Bergey: not reported)
Ammonia.............................................produced
Nitrates changed to nitrites..........................no change
Starch................................................hydrolyzed
Acid only in: lactose, glucose, raffinose, dulcitol, sucrose, mannitol, and galactose
No acid or gas in..................................salicin (Bergey: acid in salicin)
Acid and gas in........................................no sugars
Methyl Red........................................negative (Bergey: not reported)
Citrate medium...........................citrate used as source of carbon
Optimum temperature..........................25° C. to 37.5° C.
Habitat..............................................water (Bergey: soil, water, and decomposing material)

The only significant variation from Bergey's Manual is the failure of this organism to produce acid in salicin. No difficulty was experienced in identifying this organism in the keys for genera and species.

UNKNOWN NO. 19
Organism: Flavobacterium rigense. Bergey, p. 430
Shape.................................rods, with rounded ends
Size..............................................6 micron by .6 to 1.0 micron (Bergey: .75 micron by 1.75 to 2.5 microns)
Grouping...............................singly
Motility and Cilia..............................motile, flagellation not observed (Bergey: peritrichous flagella)
Gram stain.................................Gram negative
Cell contents...............................Uniform
Gelatin stab..................................yellow surface and sediment, stratiform liquefaction (Bergey: infundibuliform)
Agar plate.................................colonies white to yellow to brown, smooth
Nutrient broth...............................turbid, with pelicle; settles with age
Purple milk.................................no change
Potato..............................................yellow, turning brown; spreading
Indole..............................................no produced
Ammonia...........................................not produced
Nitrates changed to nitrites..........................changed
Starch..............................................not hydrolyzed
Acid only in..................................glucose (Bergey: no sugars)
Acid and gas in........................................no sugars
Acetyl methyl carbino........................not produced (Bergey: not reported)
Hydrogen sulfide..............................not produced
Methyl Red......................................negative (Bergey: not reported)
Citrate medium..........................citrate not used as source of carbon (Bergey: not reported)
Optimum temperature........................20° C. to 25° C. (Bergey: 30° C. to 37° C.)
Habitat..............................................water (Bergey: soil)

This organism differs from Flavobacterium in Bergey in size and growing temperature. C

This organism also as diffusum, Bergey, p. 429, ducting in gelatin, nutrient choice of Flavobacterium organism may be a variant

OBSERVATIONS

Due to the fact that Achromobacter iophagum toward the test organism included in the present paper showing the maximum dil Flavobacterium rigense incubation temperatures.

The known test organism gram positive bacteria, among the tables for better comparison

1. Bi

All broth cultures of when incubated at 37.5° C from tryptose-phosphate cultures and dilution plates toward Micrococcus catarrhales no inhibition was shown to

The tryptose-sulfite plates consistently greater productivity broth cultures. Gram negative bacteria and a few of them were not inhibited in the present paper.

These are not included in the inspection in the bound copy of the University Library.
This organism differs from the description of Flavobacterium rigens in Bergey in size, acid produced in glucose, and in optimum growing temperature. Growth of this organism was very poor at 37.5°C.

This organism also agrees to a large extent with Flavobacterium diffusum, Bergey, p. 429, except that it shows no green pigment production in gelatin, nutrient broth or on potato. This led to the writer's choice of Flavobacterium rigens, although it seems possible that this organism may be a variation of either of the species.

OBSERVATIONS AND DISCUSSION

Due to the fact that organisms Nos. 28 and 41, identified as Achromobacter tophagum, showed so little evidence of antagonism toward the test organisms, the results from their study are not included in the present paper. Tables I and II are summary tables showing the maximum dilution potency of Bacillus megatherium and Flavobacterium rigens to the known test organisms at the various incubation temperatures.

The known test organisms include four gram negative and two gram positive bacteria, and two fungi. They are shown in this order on the tables for better comparison.

1. Bacillus megatherium

All broth cultures of Bacillus megatherium showed good growth when incubated at 37.5°C, 25°C, and 20°C. Greatest inhibition from tryptose-phosphate and nutrient broth cultures when both cultures and dilution plates were incubated at 37.5°C was shown toward Micrococcus catarrhalis and Saccharomyces pastorianus, while no inhibition was shown toward Pseudomonas aeruginosa and Serratia marcescens. The tryptose-phosphate broth cultures showed a consistently greater production of antibiotic substance than did the nutrient broth cultures. Greatest inhibition was shown toward a gram negative bacterium and a fungus, while two other gram negative bacteria were not inhibited at all. Tables I-VIII of the original manuscript cover the results of B. megatherium against test organisms. These are not included in the present paper but are available for inspection in the bound copy of the original thesis in the Butler University Library.
When tryptose-phosphate and nutrient broth cultures were both incubated at 25°C and the agar dilution plates were incubated at 37.5°C, we find a variation in the maximum potency of the two broth cultures. The tryptose-phosphate broth culture showed most inhibition to *Sarcina lutea* and *Staphylococcus aureus*, while the nutrient broth culture showed most inhibition to *Micrococcus catarrhalis* and *Saccharomyces pastorianus*. The greatest inhibition was shown toward a gram positive bacterium, followed by a gram positive bacterium and a fungus, and finally a gram negative bacterium. On the other hand, *Serratia marcescens* and *Pseudomonas aeruginosa*, two other gram negative bacteria, were inhibited in low dilution at these incubation temperatures, whereas, they were not inhibited when both broth cultures and agar dilution plates were incubated at 37.5°C. There is, also, clear evidence that although the cultures incubated at 37.5°C produced more potent antibiotic substance toward some of the test organisms, the antibiotic substance was produced just as rapidly in the cultures incubated at 25°C, and it retained its potency for a longer period of time.

There was a sharp decline in the potency of the antibiotic substance produced when the tryptose-phosphate broth culture was incubated at 25°C, while the agar dilution plates were incubated at 20°C. Again, *Sarcina lutea* was inhibited in highest dilution followed by *Saccharomyces pastorianus*. No inhibition was shown toward *E. coli*, *Serratia marcescens*, and *Monilia albicans*.

When both tryptose broth culture and agar dilution plates were incubated at 20°C we find an increase in the antibiotic substance produced as compared to the results when broth cultures are incubated at 25°C and agar dilution plates were incubated at 20°C. Increased potency is evident, especially, toward *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. However, *Sarcina lutea* and *Saccharomyces pastorianus* again show the least resistance to the antibiotic substance.

A study of a *B. megatherium* culture which had been subcultured a number of times on to fresh agar slants and which was about nine weeks old when broth cultures were inoculated yielded a striking result in that the potency of the strain was reduced to one half or less during this time interval. This bears out the writer’s suspicion that the organism was losing work was being done at

In a test on the effect of *therium* a portion of the culture incubated at 37.5°C was filtered under vacuum. When the broth was incubated at 37.5°C, an inhibition in the test organism. This is that the produced had been retained with this filter and the results to continue further with

2. F.

Growth and inhibition of broth cultures of *Flavobacterium*

When both tryptose-phosphate broth cultures were incubated at 37.5°C, the maximum production of antibiotic substance occurred in the nutrient broth culture. In a test in which broth cultures were incubated at 37.5°C and agar dilution plates were incubated at 20°C, no inhibition was shown toward *E. coli*, *Serratia marcescens*, and *Monilia albicans*. However, *Sarcina lutea* and *Saccharomyces pastorianus* again showed the least resistance to the antibiotic substance.

All test organisms were...
icient broth cultures were both titrated at the highest potency of the two broth cultures, while the nutrient broth culture showed most inhibition. On 37.5°C and 20°C, the nutrient broth culture was incubated at 20°C. The maximum production of antibiotic substance was recorded. All test organisms were inhibited, and with few exceptions in fairly

the organism was losing its potency during preceding weeks when work was being done at other incubation temperatures.

In a test on the effect of filtration on a broth culture of *B. megatherium*, a portion of the original tryptose-phosphate broth culture incubated at 37.5°C was passed through an ultra-fine bacteriological filter under vacuum. The filtrate was plated in agar dilutions and incubated at 37.5°C, and there was no evidence of inhibition to any test organism. This is taken to indicate that the antibiotic substance produced had been retained on the filter. Difficulties encountered with this filter and the negative results obtained made it impractical to continue further with this procedure.

2. FLAVOBACTERIUM RIGENSE

Growth and inhibition data for known organisms tested against broth cultures of *Flavobacterium rigense* gave the following results. When both tryptose-phosphate and nutrient broth cultures and agar dilution plates were incubated at 37.5°C, lack of inhibition of any test organisms indicated that no antibiotic substance was produced in either culture. No growth was observed in either broth culture at any time during their incubation periods. When no growth occurred at the end of a five week incubation period, the cultures were discarded. Repeated inoculations of both broth media again showed no evidence of growth. Later, after several attempts, a slight growth of the Flavobacterium strain was attained on a nutrient agar slant incubated at 37.5°C. Tables IX-XV of the original thesis give the results of the tests with *F. rigense*.

On the other hand, tryptose-phosphate and nutrient broth cultures of this organism grew rapidly and vigorously at 25°C. Agar dilution plates made from these cultures showed inhibition to all test organisms when incubated at 37.5°C. The tryptose-phosphate broth culture showed a decidedly greater production of antibiotic substance than did the nutrient broth culture. *Sarcina lutea* was inhibited in highest dilution, while *E. coli* showed the greatest resistance.

When both tryptose-phosphate and nutrient broth cultures were incubated at 25°C, and the agar dilution plates were incubated at 20°C, the maximum production of antibiotic substance was recorded. All test organisms were inhibited, and with few exceptions in fairly
high dilutions. Once again, *Sarcina lutea* was inhibited in the high-
est dilution, while *E. coli* showed the greatest resistance to the anti-
biotic substance produced.

When tryptose-phosphate broth culture and agar dilution plates were incubated at 20° C. inhibition toward the test organisms was evident in high dilutions. For the most part, production of the anti-
biotic substance in the broth culture incubated at 20° C. was neither

greater nor less than production in the culture incubated at 25° C. On

the other hand, both tryptose-phosphate broth cultures, incubated at

25° C. and 20° C., showed more production of antibiotic substance

than the nutrient broth culture incubated at 25° C. The two gram

positive bacteria and the fungi were inhibited at twice the dilution in

which inhibition of the gram negative bacteria occurred.

SIGNIFICANCE OF RESULTS

Although the centrifuged culture broth medium, used in the agar
dilution plates, appeared relatively free of organisms, the incubated
agar plates showed growth of the antagonistic organisms, especially
in the lower dilutions. Consequently, the most significant observa-
tion made was that, in all cases, inhibition of the test organisms was

coincident with the growth of the antagonist on the agar dilution

plates. Furthermore, in all series but one, heavier growth of the

antagonists on the agar dilution plates, i.e., lower dilutions, resulted

in the inhibition of the maximum number of test organisms.

At first, this observation suggested a possibility that inhibition of

the test organisms was due primarily to overgrowth of the antagonist

rather than to the production of an antibiotic substance by the an-
tagont. However, further observations would seem to contradict

this contention. (1) In a number of instances, some of the test

organisms inhibited by the antagonist at one incubation temperature

were not inhibited at other incubation temperatures even though the

growth of the antagonist was as heavy or heavier in the series where

no inhibition occurred. (2) *Flavobacterium rigense* showed no evi-
dent growth in broth cultures when incubated at 37.5° C. and no

inhibition was shown to any test organisms. Furthermore, agar dilu-
tion plates incubated at 37.5° C., but prepared from culture broths

incubated at 25° C., showed a very retarded or absence of growth of

the antagonist compared to similar agar dilution plates incubated at

lower temperatures, an antibiotic substance produced at 37.5° C., showed

inhibition of both the *B. megaltherium* and *S. marcescens*, but these

organisms used.

Table I and II show the intensity of the antibiotic substances at the various incubation temperatures, in terms of the number of test organisms, as shown in these tables. *F. rigense* showed no evident growth in broth cultures incubated at 37.5° C. and no inhibition was shown to any test organisms. How-
thought inhibition of the antibiotic substances produced under all conditions are indicated in these tables.

*B. megaltherium*, cultures incubated at 37.5° C. showed no antibiotic substance produced at 37.5° C., but showed the greatest inhibition of the test organisms at 25° C. and 20° C.

At 37.5° C. incubation temperatures, an antibiotic substance prepared from culture broths prepared at 25° C., showed a very retarded or absence of growth of the antagonist compared to similar agar dilution plates incubated at 25° C., but prepared from culture broths incubated at 25° C., showed a very retarded or absence of growth of the antagonist compared to similar agar dilution plates incubated at
Bacteria was inhibited in the highest dilution toward the test organisms was at 37.5°C. Inhibition of the test organisms was observed in both culture and agar dilution plates. Inhibition toward the test organisms was greatest in the agar dilution plates, incubated at 20°C. On the other hand, inhibition was observed in the agar dilution plates, incubated at 25°C C. The two gram negative bacteria were inhibited at twice the dilution in which inhibition was observed.

**Summary of Results**

The culture broth medium, used in the agar dilution plates, was incubated at 20°C. Production of antibiotic substance occurred at 25°C. The two gram negative bacteria were inhibited at twice the dilution in which inhibition was observed.

In the writer's opinion, the observations cited above indicate that both the *B. megaltherium* and *F. rigense* produced antibiotic substances, but these substances were not separated from the organisms. Possibly other methods of treatment might have accomplished this separation.

Tables I and II are summary tables showing the maximum potency of the antibiotic substances produced in the culture broth media at the various incubation temperatures. Potency figures are expressed in terms of the maximum dilution at which inhibition of the test organism occurred.

As shown in the tables, these strains of *B. megaltherium* and *F. rigense* showed rather surprising antagonism to all of the eight test organisms used. Reference to other bacteria producing antibiotic substances show that they generally tend to be specific toward certain organisms. However, the tables show clear evidence that, even though inhibition was shown to all test organisms, actually the antibiotic substances produced were specific for certain organisms when all conditions are taken into consideration.

*B. megaltherium* showed production of an antibiotic substance at all incubation temperatures with the greatest potency occurring in cultures incubated at 37.5°C. On the other hand, *F. rigense* showed no antibiotic substance produced at 37.5°C, but it produced a more potent antibiotic substance than *B. megaltherium* when cultured at 25°C and 20°C.

At 37.5°C incubation temperature, *B. megaltherium* produced the greatest inhibition to *Micrococcus catarrhalis*, a gram negative bacterium, and to *Saccharomyces pastorianus*, a fungus, while at 25°C and 20°C, the greatest inhibition produced by *B. megaltherium* was toward *Sarcina lutea*, a gram positive bacterium. On the other hand, *Serratia marcescens*, *Pseudomonas aeruginosa* and *E. coli*, all gram negative bacteria, showed the greatest resistance to the antibiotic substance produced by the *B. megaltherium*.

*F. rigense* produced, at both 25°C and 20°C incubation temperatures, an antibiotic substance showing greatest inhibition to *Saccharomyces pastorianus*. Nevertheless, the agar dilution plates incubated at 37.5°C, showed inhibition to all test organisms, and all, except *E. coli*, were inhibited in dilution of 1:100 or more.
*Cma lutea* and *Staphylococcus aureus*, both gram positive bacteria, while the least inhibition was shown to *Serratia marcescens* and *E. coli*, both gram negative bacteria.

In general, both antagonists showed greatest inhibition to the gram positive organisms, while the gram negative organisms showed the most resistance.

Table III shows the period, in number of days, when the greatest amount of antibiotic substance was produced in the various culture broths.

Tryptose-phosphate broth cultures of *B. megatherium* showed the greatest production of antibiotic substance between 5-8 days when both broth culture and agar dilution plates were incubated at 37.5° C.; between 11-17 days when the broth culture was incubated at 25° C., and the agar dilution plates were incubated at 37.5° C.; between 9-16 days when the broth culture was incubated at 25° C., and the agar dilution plates were incubated at 20° C.; and between 3-8 days when both broth culture and agar dilution plates were incubated at 20° C.

Nutrient broth cultures of *B. megatherium* showed the greatest production of antibiotic substance between 9-14 days when both broth culture and agar dilution plates were incubated at 37.5° C.; and between 9-16 days when the broth culture was incubated at 25° C., and the agar dilution plates were incubated at 20° C.

Tryptose-phosphate and nutrient broth cultures of *F. rigense* showed no production of antibiotic substance when both broth cultures and agar dilution plates were incubated at 37.5° C. Tryptose-phosphate broth cultures showed the greatest production of antibiotic substance between 6-15 days when the broth culture was incubated at 25° C., and the agar dilution plates were incubated at 37.5° C.; between 6-25 days when the broth culture was incubated at 25° C., and the agar dilution plates were incubated at 20° C.; and between 3-17 days when both broth culture and agar dilution plates were incubated at 20° C.

Nutrient broth cultures of *F. rigense* showed the greatest production of antibiotic substance between 3-16 days when the broth culture was incubated at 25° C., and the agar dilution plates were incubated at 37.5° C.; and between 6-25 days when the broth culture was incubated at 25° C., and the agar dilution plates were incubated at 20° C.

*F. rigense* was not affected by prolonged culturing on *B. megatherium*.

One of the test organisms showed particular evidence of *F. rigense* producing antibiotic substance at incubation temperatures of 37.5° C. Tryptose-phosphate broth cultures produced the greatest amount of antibiotic substance at 25° C. and 20° C.

1. Bacteria producing antibiotic substance at incubation temperatures of 37.5° C. and 25° C.
2. *B. megatherium* produced the greatest amount of antibiotic substance at incubation temperatures of 37.5° C. and 25° C.
3. *F. rigense* produced the greatest amount of antibiotic substance at incubation temperatures of 25° C. and 20° C.
4. *Achromobacter* produced the greatest amount of antibiotic substance at incubation temperatures of 37.5° C. and 25° C.
5. *F. rigense* produced the greatest amount of antibiotic substance at incubation temperatures of 25° C. and 20° C.
was incubated at 25° C, and the agar dilution plates were incubated at 20° C.

*B. megatherium* apparently lost some of its potency through prolonged culturing on artificial media, while the potency of the *F. rigense* was not effected noticeably.

One of the test organisms, *Saccharomyces pastorianus*, showed particular evidence of being a weak strain through the culture series. The other test organisms consistently showed fairly vigorous growth, except in rare instances.

**CONCLUSIONS**

1. Bacteria producing antibiotic substances were isolated from the White River Canal, Indianapolis, Indiana.
2. These organisms have been identified tentatively as *Achromobacter iophagum*, *Bacillus megatherium*, and *Flavobacterium rigense*.
3. To the writer’s knowledge no production of antibiotic substance has been reported for any of these organisms.
4. *Achromobacter iophagum* showed inhibition to only one of eight test organisms in low dilutions. On the other hand *B. megatherium* and *F. rigense* produced inhibition to most or all of the test organisms; in some cases, in fairly high dilutions.
5. *F. rigense* produced an antibiotic substance which was more potent to the eight test organisms than that produced by *B. megatherium*.
6. Broth cultures of *B. megatherium* produced the antibiotic substance at incubation temperatures of 37.5° C, 25° C, and 20° C. Tryp­tose-phosphate broth cultures produced more antibiotic substance than did the nutrient broth cultures, with greatest production occurring when both broth cultures and agar plates were incubated at 37.5° C.
7. Broth cultures of *F. rigense* produced antibiotic substance at incubation temperatures of 25° C and 20° C, while none was produced at 37.5° C. Tryp­tose-phosphate broth cultures produced greater amounts of antibiotic substance than the nutrient broth cultures with maximum production occurring when the broth culture was incubated at 25° C, and the agar dilution plates were incubated at 20° C.
8. *B. megatherium* showed evidence of losing its potency after successive culture and incubation periods, while *F. rigens*e showed no evidence of loss of potency.

9. No attempt was made to isolate the antibiotic substances from the organisms.

ACKNOWLEDGMENT

The writer wishes to express his sincere appreciation to Dr. Ray C. Friesner for suggesting the problem and critical reading of the manuscript, and to William A. Daily and Fay Kenoyer Daily for their many helpful suggestions during the course of the research.

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### TABLE I

**Bacillus megatherium**

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<th>Culture Media</th>
<th>Conditions of Culture</th>
<th>Temperature Centigrade</th>
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### TABLE II

**Flavobacterium rigense**

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<tr>
<td>Tryptose-Phosphate Broth</td>
<td>centrifuged</td>
<td>20</td>
<td>20</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>200</td>
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</tr>
</tbody>
</table>

**TABLE II**

Flavobacterium rigense

Maximum Potency of Agar Dilution Units Versus Test Organisms
### TABLE III

Occurrence of Maximum Production of Antibiotic Substance

<table>
<thead>
<tr>
<th>Culture Broth Medium</th>
<th>Incubation Temperature of Culture Broth °C.</th>
<th>Incubation Temperature of Agar Plates °C.</th>
<th>Bacillus megaterium</th>
<th>Flavobacterium rigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose-Phosphate Broth</td>
<td>37.5</td>
<td>37.5</td>
<td>5-8</td>
<td></td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>37.5</td>
<td>37.5</td>
<td>9-14</td>
<td></td>
</tr>
<tr>
<td>Tryptose-Phosphate Broth</td>
<td>25</td>
<td>37.5</td>
<td>11-17</td>
<td>6-15</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>25</td>
<td>37.5</td>
<td>9-16</td>
<td>3-16</td>
</tr>
<tr>
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<td>25</td>
<td>20</td>
<td>9-16</td>
<td>6-25</td>
</tr>
<tr>
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<td>9-16</td>
<td>6-25</td>
</tr>
<tr>
<td>Tryptose-Phosphate Broth</td>
<td>20</td>
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<td>3-8</td>
<td>3-17</td>
</tr>
</tbody>
</table>

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GROWTH CORRECTION

DECIDUOUS TREES

B

Since the beginning of wood science as a science, work on radial growth has been centered around growth studies that were carried out in America on trees. Such work on radial growth is less extensive on the deciduous tree. Dendrometer studies of radial growth in deciduous trees have been carried out by Beilmann and recently by Peuhkuri (7). Recently Daubenmire (13) (18) (19) (29).

Axial growth behavior has been studied in a number of species of deciduous trees. Axial growth may be divided into two types: (a) short season growth that occurs in 30 days; and (b) long season growth that occurs in 60 days and the trees of both groups have been studied. The species of deciduous trees that belong to the short season group are Acer rubrum, Fagus grandifolia, and P. sylvestris. The species of deciduous trees that belong to the long season group are Tilia americana, Acer saccharum, and others. (15, 16) has shown that P. sylvestris belong to the short season group and Tilia americana Fagus grandifolia belong to the long season group.

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* A portion of a thesis for the Master of Arts degree at the University.