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

Fred C. Mindach

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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 Daudenmire, 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.
ANTIBIOTIC PRODUCING ORGANISMS ISOLATED FROM THE WHITE RIVER CANAL, INDIANAPOLIS, 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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4 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 Giacca (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 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 food-stuffs. 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 iophagum*, 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 *Bacillus megaterium* and *Flavobacterium nigense*. 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.

Formulæ for these:

| Bacto-Beef Extra | Bacto-Peptone | Distilled Water |
| Bacto-Dextrose | Bacto-Peptone | KH,PO₄ |
| MgSO₄ . 7 H₂O | FeSO₄ . 7 H₂O | Bacto-Agar |
| Distilled Water |

The bacterial count follows: October 27, colonies per ml; and November 4, November 8, the canal water* show colonies, a minimum of per ml. Temperatures of 56°F., and 46°F., respectively, was 55°F.

The agar dilution plates of C-D 1 agar were incubated at 37°C. until good growth developed. Only well isolated colonics were selected. Once again a well isolated culture was transferred to a test tube agar slant of C-D 2 (synthetic) Czapek-Dox medium.

| Bacto-Beet | Bacto-Dextrose | NaNO₃ | KH.PO₄ |

* Taken from the American Type Culture Collection.
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 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.

Formulae for these media are as follows.

**NUTRIENT AGAR**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Beef Extract</td>
<td>3 gm</td>
</tr>
<tr>
<td>Bacto-Peptone</td>
<td>5 gm</td>
</tr>
<tr>
<td>Bacto-Agar</td>
<td>15 gm</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

**C-D 1 AGAR**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Dextrose</td>
<td>20 gm</td>
</tr>
<tr>
<td>Bacto-Peptone</td>
<td>5 gm</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>MgSO₄ - 7 H₂O</td>
<td>0.25 gm</td>
</tr>
<tr>
<td>Fe SO₄ - 7 H₂O</td>
<td>0.01 gm</td>
</tr>
<tr>
<td>Bacto-Agar</td>
<td>15 gm</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

* Taken from the Annual Laboratory Report of the Indianapolis Water Company.

25
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**

- Bacto-Tryptose: 20 g
- Bacto-Dextrose: 2 g
- Sodium Chloride: 5 g
- Disodium Phosphate: 2.5 g
- Distilled Water: 1 liter
- 15% Bacto-Agar (added for broth enrichment)
- 2.0% Bacto-Agar (added for solid medium)
The organisms on nutrient agar were grown in order to prove their ability to grow on the agar. A total of sixty-seven organisms were selected as the test organisms. All but four appeared to be bacteria. Due to an unavoidable lapse in time, some of the cultures were lost.

For the forty-five cultures remaining, plates of agar (pH 6.8) and also on C-D 2 were prepared. At room temperature until good growth appeared. Then approximately 20% catarhalis, E. coli, Staphylococcus aureus were made at right angles to and in the unknown organisms. The room temperature for 96 hours and no evidence of inhibition.

At the forty-five unknown organisms, or more of the known test organisms. 10 and 19, isolated from the water on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and in the unknowns Nos. 28 and 41, isolated on November 4, 1947. Inhibition zones were made at right angles to and 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. The dilutions were made as agar dilution units; i.e., 2 ml of culture broth plus 18 ml of agar represented a 1:100 dilution, etc. 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.

### NUTRIENT MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Beef Extract</td>
<td>3 gm</td>
</tr>
<tr>
<td>Bacto-Peptone</td>
<td>5 gm</td>
</tr>
<tr>
<td>15% Bacto-Agar (added for broth enrichment)</td>
<td>1 liter</td>
</tr>
<tr>
<td>20% Bacto-Agar (added for solid medium)</td>
<td>1 liter</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 lb. 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 20% 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. The dilutions were made as agar dilution units; i.e., 2 ml of culture broth plus 18 ml of agar represented a 1:100 dilution, etc. 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.

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**OSSPHATE MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% Bacto-Beef Extract</td>
<td>20 gm</td>
</tr>
<tr>
<td>2% Bacto-Peptone</td>
<td>2 gm</td>
</tr>
<tr>
<td>5% Bacto-Agar</td>
<td>5 gm</td>
</tr>
<tr>
<td>2.5% Bacto-Agar (added for broth enrichment)</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>1% Bacto-Agar (added for solid medium)</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

---

27
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 temperatures. In this way, known organisms were added to the products for the production of the final product. When C. they were allowed to incubate for an additional 24 hours, and the organisms, with the exception of those in dilution series plates after 24 hours' incubation, no agar dilution plates were made.

The strains of the Erlich race were E. coli, E. catharalis (Lilly), E. c. Monilia albicans (Lilly), and Mononas aeruginosa (Butler). The strains of the Erlich race were E. coli, E. catharalis (Lilly), E. c. Monilia albicans (Lilly), and Mononas aeruginosa (Butler).

IDENTIFICATION

Bergey's Manual of Determinative Bacteriology used for the identification of the products are cited, and discussions of the strains of the Erlich race were E. coli, E. catharalis (Lilly), E. c. Monilia albicans (Lilly), and Mononas aeruginosa (Butler).

Organism: Bacillus

Shape
Size
Grouping
Motility and Cilia
Gram stain
Spores
paraterminal, 8 to 1.0 m
Cell contents
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), *Mona lisa albus* (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

<table>
<thead>
<tr>
<th>Shape</th>
<th>Shape rods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>1.5 microns by 3.0 to 5.0 microns</td>
</tr>
<tr>
<td>Grouping</td>
<td>singly, pairs, short chains</td>
</tr>
<tr>
<td>Motility and Cilia</td>
<td>Motile, flagellation not observed (Bergey: peritrichous flagella)</td>
</tr>
<tr>
<td>Gram stain</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Spores</td>
<td>paracentral to paraterminal, .8 to 1.0 micron by 1.0 to 1.5 microns, cylindrical, not swollen</td>
</tr>
<tr>
<td>Gelatin stab</td>
<td>slow liquefaction, crateriform to infundibuliform</td>
</tr>
<tr>
<td>Agar plate</td>
<td>creamy white, moist, with pellucid dots</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>heavy uniform turbidity</td>
</tr>
</tbody>
</table>

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 0.8 to 1.0 micron (Bergey: 0.75 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)
- Nutrient broth: colonies white to yellow to brown, smooth
- Agar plate: colonies growth, turbid, with pellicle: settles with age
- Purple milk: no change
- Potato: yellow, turning brown; spreading
- Indole: not produced
- Nitrites changed to nitrites: no change
- Starch: not hydrolyzed
- Acids only in: glucose (Bergey: no sugars)
- Acid and gas in: no sugars
- Acetate: methyl carbino: not produced (Bergey: not reported)
- Hydrogen sulfide: not produced (Bergey: not reported)
- 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 *Bergey* in size growing temperature: C 37.5° C.

This organism also as *diffusum*, Bergey, p. 429, due to the presence of nitrites and the nitrites to nitrites in incubation temperatures.

The known test organism is gram positive bacteria, an on the tables for better co

1. Bi

All broth cultures of *Bergey* when incubated at 37.5° C from tryptose-phosphate cultures and dilution plates toward *Micrococcus catarh* no inhibition was shown to *marcescens*. The tryptose consistently greater production of trioent broth cultures. Gram negative bacterium and a *Bergey* were not inhibited at incubation temperatures. These are not included in script cover the results of these observations in the bound copy.


This organism differs from the description of Flavobacterium rigense 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 rigense, 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 iophagum, 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 rigense 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 *Mollisia 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 37.5°C. In a test on the effect of *therium* a portion of the culture incubated at 37.5°C was filter under vacuum. This culture was incubated at 37.5°C. This is 1 produced had been retained with this filter and the results of the tests with this culture are shown in Table 1.

2. Flavobacterium sp.

Growth and inhibition of broth cultures of *Flavobacterium*. When both tryptose-phosphate and nutrient broth cultures were inoculated on to fresh agar slants and the agar dilution plates were incubated at 37.5°C, growth and inhibition plates made from these cultures showed some indications of the growth of the Flavobacterium sp. All test organisms were inoculated on to these plates. The results of the tests with this organism showed a decided inhibition of the test organisms indicated in either culture. No growth was noted at the end of a five week period. Repeated inoculation produced no evidence of growth. Lack of growth indicates that this organism was not strain of the Flavobacterium sp. All test organisms were inoculated on to these plates. The results of the tests with this organism showed a decided inhibition of the test organisms indicated in either culture. No growth was noted at the end of a five week period. Repeated inoculation produced no evidence of growth. Lack of growth indicates that this organism was not
ient broth cultures were both at mum potency of the two broth cultures showed most inhibitory action on *Micrococcus catarrhalis* and the least inhibition was shown toward *Sarcina lutea*. While the nutrient broth culture showed most inhibitory action on *Staphylococcus aureus*, the nutrient agar dilution plates were incubated at 37.5°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 dilution, 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 37.5°C., the maximum production of antibiotic substance was recorded. All test organisms were inhibited, and with few exceptions in fairly high dilution, the antibiotic substance was produced just as it was when broth cultures were incubated at 37.5°C. and it retained its potency when broth cultures were incubated at 20°C. and agar dilution plates were incubated at 20°C. Potency of the antibiotic substance was lost when broth cultures were incubated at 20°C. and agar dilution plates were incubated at 20°C.
high dilutions. Once again, *Sarcina lutea* was inhibited in the highest dilution, while *E. coli* showed the greatest resistance to the antibiotic 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 antibiotic 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 observation 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 antagonist. 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) *Fusobacterium rigenense* showed no evident growth in broth cultures when incubated at 37.5° C. and no inhibition was shown to any test organisms. Furthermore, agar dilution 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, at 37.5° C., showed no inhibition of *E. coli*, were inhibited by the antagonist.

In the writer’s opinion, both the *B. megalatherium* and the *F. rigenense* showed no evidence of the inhibition of the test organisms used. Substances show the production of potent antibiotic substances at 25° C. and 20° C. In all series but one, heavier growth of the antagonist on the agar dilution plates, i.e., lower dilutions, resulted in the inhibition of the maximum number of test organisms.

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In the writer’s opinion, both the *B. megalatherium* and the *F. rigenense* showed no evidence of the inhibition of the test organisms used. Substances show the production of potent antibiotic substances at 25° C. and 20° C. In all series but one, heavier growth of the antagonist on the agar dilution plates, i.e., lower dilutions, resulted in the inhibition of the maximum number of test organisms.
Cina lutea was inhibited in the highest degree by the agar dilution plates, but with agar dilution plates, the greatest inhibition to the test organisms was produced at 37.5°C and 20°C. 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. megalhcrium 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. megalhcrium.

F. rigense produced, at both 25°C and 20°C incubation temperatures, an antibiotic substance showing greatest inhibition to Sar-
cinna 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 37.5° 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.; and 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 37.5° C.; and between 3-17 days when both broth culture and agar dilution plates were incubated at 20° C.

**B. megatherium** antagonist produced potent antibiotic substance at incubation temperatures occurring when both broth cultures and agar dilution plates were incubated at 37.5° C.

1. Bacteria producing antibiotic substances at the White River Canal,
2. *B. megatherium* and *F. rigense* are potent to the eight test organisms; in some cases, the potency was increased when both broth cultures and agar dilution plates were incubated at 37.5° C.

**F. rigense** produced potent antibiotic substance at incubation temperatures occurring when both broth cultures and agar dilution plates were incubated at 37.5° C.

### Table III

<table>
<thead>
<tr>
<th>Culture Type</th>
<th>Incubation Temperature</th>
<th>Maximum Production of Antibiotic Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose-phosphate</td>
<td>37.5° C, 25° C</td>
<td>Variable</td>
</tr>
<tr>
<td>Nutrient</td>
<td>37.5° C, 25° C</td>
<td>Variable</td>
</tr>
<tr>
<td>Both</td>
<td>37.5° C, 25° C</td>
<td>Variable</td>
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</tbody>
</table>

One of the test organisms produced potent antibiotic substance at incubation temperatures occurring when both broth cultures and agar dilution plates were incubated at 37.5° C.
B. megatherium showed the greatest inhibition to the negative organisms showed in the various culture of days, when the greatest

37.5° C; and 20° C. 

B. megatherium showed between 5-8 days when both

37.5° C; and 20° C. 

B. megatherium showed the greatest 9-14 days when both

37.5° C; and 20° C. 

Achromobacter iophagum showed inhibition to only one of eight test organisms in low dilutions. On the other hand B. megatherium and F. rigens produced inhibition to most or all of the test organisms, in some cases, in fairly high dilutions. 

F. rigens produced an antibiotic substance which was more potent to the eight test organisms than that produced by B. megatherium.

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.

F. rigens 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. rigense* 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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15. Zobell, C. E. Bacterial 34:113-116. 1936. (Ci
of losing its potency after
while F. rigensae showed
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Kenoyer Daily for their

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2. VACEK, B. Examination of some conditions necessary for the survival of the typhoid bacillus in water. Water Pollution Research, 6:272-273. 1933. (Cited by Waksman, 13.)
TABLE I
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Maximum Potency of Agar Dilution Units Versus Test Organisms

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<thead>
<tr>
<th>Culture Media</th>
<th>Condition of Culture</th>
<th>Incubation Temperature C</th>
<th>Temperature of Agar Plate C</th>
<th>Micrococcus casei</th>
<th>Escherichia coli</th>
<th>Pseudomonas aerugiosa</th>
<th>Staphylococcus aureus</th>
<th>Staphylococcus pyogenes</th>
<th>Streptococcus faecalis</th>
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TABLE II
Flavobacterium rigense
Maximum Potency of Agar Dilution Units Versus Test Organisms

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<thead>
<tr>
<th>Culture Media</th>
<th>Condition of Culture</th>
<th>Incubation Temperature C</th>
<th>Temperature of Agar Plate C</th>
<th>Micrococcus casei</th>
<th>Escherichia coli</th>
<th>Pseudomonas aerugiosa</th>
<th>Staphylococcus aureus</th>
<th>Staphylococcus pyogenes</th>
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**TABLE II**

*Flavobacterium rigense*

Maximum Potency of Agar Dilution Units Versus Test Organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram Negative</th>
<th>Gram Positive</th>
<th>Fungi</th>
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<tr>
<td>Micrococcus Aureus</td>
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<td>Escherichia coli</td>
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<td>Saccharomyces cerevisiae</td>
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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 megatherium</th>
<th>Flavobacterium rigens</th>
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</table>

GROWTH CORRELATION DECIIDUOUS TREES

Since the beginning of work on radial growth in deciduous trees, the work on radial growth has been extensively reviewed. (28) The data compiled by Bellman (7) are too extensive for a summary here. Recently Daubenmire and Beilman (5) have published the results of drometer studies on deciduous and coniferous trees. The compilation of data on radial growth studies has been made by Beilman (13), (18), (19), (29).

Axial growth behavior has been studied in a number of species. It may be divided into two major groups. One group comprises the short season group and the other the long season group. The short season group includes trees that grow quickly in the spring and summer and then cease growth. The long season group includes trees that grow slowly throughout the year.

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