

ACID-TOLERANT, GRAM-NEGATIVE BACTERIA  
ISOLATED FROM MONTANAN CONIFEROUS BED  
AND FIELD SOIL SAMPLES

DATE

Submitted in Partial Fulfillment of the Requirements for  
Graduation with Honors to the Department of Biology at  
Carroll College, Helena, Montana

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March 22, 1983

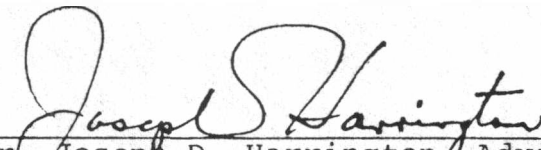
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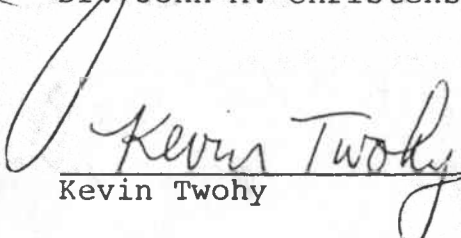
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## ABSTRACT

Montanan field soil was found to have a pH of approximately 8.5 and coniferous bed soil a pH of approximately 7.0. From these soils, 10 Gram-negative bacilli were isolated by using selective, low pH media.

The two soils yielded different isolates. Two isolates were identified as members of the family Enterobacteriaceae, probably of the genus Erwinia. The other isolates could not be identified.

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## INTRODUCTION

Numberous bacteria live in the soil. These organisms are vital to the ecology of the biosphere because they play a large role in the cycling of the major bio-essential elements i.e., carbon, nitrogen, phosphorus, sulfur, oxygen and hydrogen.

Part of the soil bacterial population has the ability to grow in an acidic environment, as well as the ability to grow in an alkaline environment, as the typical soil seen in Montana. Most of these diverse bacteria are Gram-negative, rod-shaped, facultative anaerobes (4).

The purpose of this research is to study these Gram-negative, rod-shaped, facultative anaerobes to gain more insight on their characteristics. Also, an attempt was made to identify these organisms.

## LITERATURE REVIEW

### SOIL FORMATION

The formation of the mineral part of the soil begins with the weathering of rocks. It involves hydrolysis, hydration, oxidation, carbonation and solution. The genesis of soil really begins when organisms invade this mineral substance and begin to accumulate organic substances on the surface. The mere physical and chemical weathering of rocks should not be confused with soil formation, but thought of as only part of the process (8).

### SOIL FRACTIONS

Soil is composed of four fractions that are finely subdivided and intimately mixed. These fractions are mineral materials, organic matter, water and air (8).

One part of the soil is the mineral materials. The mineral constituents of the soil have their origins from the rocks that are common to the earth's surface. Some of these rocks are limestone, granite, sandstone and shale (8). The dominate mineral particles in most soils are compounds of silicon, aluminum, iron and lesser amounts of other minerals, including calcium, magnesium, potassium, titanium, manganese, sodium, nitrogen, phosphorus and sulfur (10).

Another part of the soil is the organic fraction. The organic component of soil is small, (about 4% in representative mineral soil), but it is still vital. It is necessary for soil formation. Also, most of the nitrogen

and sulfur of soil are locked up in organic combinations. Organic matter helps to granulate the soil. It also increases the water-holding capacity. Most of the organic matter is the main energy source for soil micro-organisms (8).

A third fraction is the water portion. The water portion of soil depends on soil composition, the living population of the soil and precipitation and other climatic conditions. Water is retained as free H<sub>2</sub>O in the intra-soil spaces, which are between soil particles. Water adsorbs onto the surface of the soil particles. Soil water is important because inorganic and organic components of soil are dissolved in soil water, which makes them available as nutrients for soil inhabitants (10).

The last fraction of the soil is the air fraction. Soil air, unlike atmospheric air, is highly dispersed within the soil. Soil air is adsorbed by the soil colloids, and an appreciable amount is dissolved in the soil water. Soil air, like atmospheric air, contains a mixture of nitrogen, oxygen, carbon dioxide and certain minor gases. These gases, though, are in different proportions in soil air compared to soil air. Carbon dioxide is more plentiful in the soil air because it is liberated in the decay of organic matter. This carbon dioxide dominates the soil air. As the carbon dioxide levels go up, the nitrogen and oxygen levels go down; this favors anaerobic conditions (8).

#### SOIL PROFILES

To understand a type of soil, one should know the pro-

file of the soil. A soil profile is a succession of layers in a vertical section down into loose weathered rock. The profile nature depends on the growth of roots, the storage of moisture and the supplies of plant nutrients. The profile consists of at least two layers which are parallel to the land surface. These layers are known as horizons. Horizons differ in one or many properties, such as thickness, porosity, texture or structure (13). A hypothetical profile is demonstrated in Table 1.

#### CONIFEROUS BED SOIL

Coniferous bed soil is best classified as podzolic soil. Podzolic soil commonly has distinct A2 horizons (Table 1). Some of the podzolic soils have B horizons which are accumulations of sesquioxides (an oxide containing three oxygen atoms and two atoms of a different element, or two radicals), humus, or both. While others have B horizons that are mainly accumulations of clay with small amounts of humus and sesquioxides. Coniferous bed soil is commonly acidic, low in bases such as calcium, and low in organic matter (5,13).

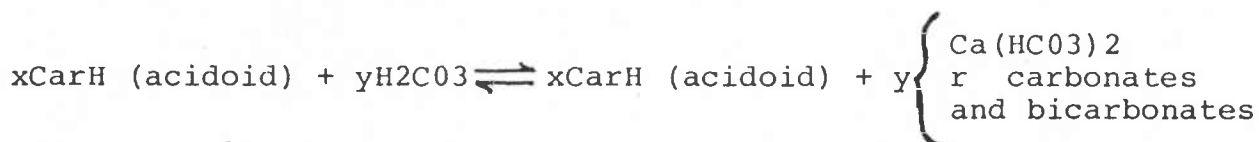
#### FIELD SOIL

Field soil is best classified as chernozemic soil. Chernozemic soil normally has dark A1 horizons of great thickness (Table 1). The B horizons usually are much less distinct. The chernozemic soil profiles compare to those of the podzolic soils in depth, but this is all they have in common. Chernozemic soils are higher in bases, less

acidic, and higher in plant nutrients compared to the podzolic soil (13).

#### SOIL ACIDITY

One of the differences between the podzolic and the chernozemic soils is pH. The main factor that effects pH is the carbon dioxide in the soil. Since carbon dioxide develops rapidly, carbonic acid forms, which raises the hydronium ion concentration. The generalized reaction is as follows:



If the soluble bicarbonates and carbonates are removed, as in the case of leaching that occurs prominently in podzolic soil, then the reaction will move to the right. The colloidal complexes steadily lose their adsorbed metallic cations, which lowers the base saturation and raises the hydronium ion concentration (8).

#### IMPORTANCE OF SOIL BACTERIA

Soil bacteria play an important role in the ecology of the biosphere. They act as biogeochemical agents that mineralize organic carbon, nitrogen, sulfur, phosphorus and other compounds as well. Since the earth has a finite amount of these essential elements, they must be used and re-used. Bacteria help bring about this cycling (10).

Soil bacteria also are important in the rhizosphere. The rhizosphere is the region where the soil and the roots of plants make contact. More soil bacteria exist in this

environment than in a root-free soil. The reason is because a degree of symbiosis exists between the plants and the bacteria. Bacterial growth is enhanced by nutrients released from the plant tissue. Some of these substances are amino acids and vitamins. The products of the microbial metabolism released into the soil, in turn, often promote the growth of the plant (10).

Another important aspect of the soil bacteria is that they play an important role in the formation of the humus. When the remains of dead animals and dead plants fall upon the ground, they undergo decomposition. Some parts of these remains are not rapidly digested. This material accumulates in the soil. As further decomposition occurs, the residues are converted into humus. Humus is a source of essential plant food (2).

#### CHARACTERISTICS OF SOIL BACTERIA

Soil bacteria are numerous in the soil. Direct microscope counts have reported that the number of bacteria in the soil is as high as several billion per gram. All types of bacteria exist in the soil; autotrophs, heterotrophs, mesophiles, thermophiles, psychrophiles, anaerobes, aerobes, sulfur oxidizers, cellulose digesters, protein digesters and nitrogen fixers (10).

Rod shaped bacteria, less than one micron wide and a few microns long, are the most common in the soil. Many are motile by means of peritrichous or polar flagella.

Also, many are capsulated (6). Depending on the soil location, it is estimated that from 5 to 35 percent of soil bacteria are of the genus Arthrobacter. Pseudomonas, Clostridium, Bacillus, Micrococcus, and Flavobacterium are some of the other common soil bacteria (9).

Bacterial growth in the soil takes place mainly on soil crumbs. Here the bacteria aggregate into microcolonies, clusters of approximately 10 bacterial cells. The bacteria are not distributed uniformly throughout the soil. They predominate where the organic matter is, which is the upper soil layers or topsoil (9).

#### EFFECT OF PH ON BACTERIA

The pH range that most bacteria can grow in is pH 4 to pH 9 (3). The optimum pH range for optimum growth, though, is pH 6.5 to pH 7.5. The specific effect of the hydronium ion concentration of the soil on bacteria is not known. The hydronium ion concentration may have a direct affect on the cell or it may effect the cell by changing the availability of the various organic and inorganic nutrients. There is not much information on the separation of these two hydrogen ion effects (4).

The hydrogen ion concentration may have a direct effect on the bacterial cell by affecting the activity of enzymes. Each enzyme has maximal activity at a certain pH, and this activity declines sharply on each side of the pH value (7).

#### HISTORY OF BACTERIAL CLASSIFICATION

In early bacterial classification, bacteria were

classified in a natural system, a system which reflects the relationships between organisms on the basis of their probable origin. Opposed to the natural system is an artificial system, which is based on easily recognizable properties of an organism (14). Bacteria were first thought to be part of the animal system. Later, bacteria were thought to be part of the plant kingdom and were put in the class Schizomycetes (14).

In 1923, the first edition of Bergey's Manual of Determinative Bacteriology was published by the Society of American Bacteriologists (now the American Society for Microbiology). Since 1923, seven additional editions have been published. The book has become the best known modern text on bacterial classification (14).

Before 1974, Bergey's Manual of Determinative Bacteriology used a natural system of classification. The eighth edition is based more on an artificial system. Since more knowledge of bacteria has accumulated, natural relationships are now thought to be difficult to establish.

The eighth edition contains 19 parts which are based on a few easily determined criteria. There is some hierarchy taxonomy such as order, family, genus, species for some types of bacteria, though (1).

#### IDENTIFICATION

A basis for identification of most common bacteria is the morphology of the individual organism, its staining reactions, and characteristic colony formation.

Although morphology and staining reactions provide certain information important to the classification and identification of bacteria, by themselves, they are inadequate and must be supplemented by other properties, such as biochemical activity, antigenic structure and pathogenicity (12).

Table 1 . Idealized Soil Profile.

- A00 Loose leaves and organic debris, largely undecomposed.
- A0 Organic debris partially decomposed or matted.
- A1 A dark-colored horizon with a high content of organic matter mixed with mineral matter.  
A light-colored horizon of maximum eluviation. Prominent in Podzolic soils; faintly developed or absent in Chernozemic soils.
- A3 Transitional to B, but more like A than B. Sometimes absent.
- B1 Transitional to B, but more like B than A. Sometimes absent.
- B2 Maximum accumulation of silicate clay minerals or of iron and organic matter; maximum development of blocky or prismatic structure; or both.
- B3 Transitional to C.
- C Horizon C for intensely gleyed layers, as in hydromorphic soils.
- Coa C  
Cos Horizons Coa and Cos are layers of accumulated calcium carbonate and calcium sulfate found in some soils.

## MATERIALS AND METHODS

On September 4, 1982, 10 soil samples were collected from hills about 12 mi northeast of Helena, Montana. The soil temperature was recorded. The top 1 in of the soil, from which the sample was to be taken, was scraped away with a metal digging instrument. The instrument was shoved into the soil a few times in the digging site to remove foreign bacteria. The soil was put into a Whirl-Pak bag. The bags were sealed and labeled.

Ten ml of each soil sample were diluted with 90 ml of sterile distilled water. Ten ml of each diluted soil sample were added to modified double strength Minimal Broth Davis, pH1. PH of the broth was lowered by adding H<sub>2</sub>S0<sub>4</sub>. PH was tested with litmus paper. Ten ml of each diluted soil sample were added to modified double strength Sabouraud Dextrose Broth, pH1. Also, 1 ml of each diluted soil sample was added to modified normal strength Minimal Broth Davis, pH1, and 1 ml of each diluted soil sample was added to modified normal strength Sabouraud Dextrose Broth, pH1. Finally, 0.1 ml of each diluted soil sample was added to modified normal strength Minimal Davis Broth, pH1, and 0.1 ml of each diluted soil sample was added to modified normal strength Sabouraud Dextrose Broth, pH1. These inoculated broths were then incubated at 23 C for 5 weeks.

The pH of the soil was determined by adding 20 gm of soil to 100 ml of aerated distilled water, then mechanically

shaking the mixture for 1 hr, followed by testing the mixture with a pH meter with a glass/calomel electrode.

The remnants of the soil samples were frozen at -24 C.

The frozen soil samples were removed from the freezer and allowed to thaw. When the samples were at room temperature, 10 ml of the soil were removed. The remnants were refrozen. The removed 10 ml of soil was diluted with 90 ml of sterile distilled water. Two-tenths ml of each diluted soil sample were used to inoculate plates of modified Sabouraud agar, pH3, and plates of modified Minimal Agar Davis, pH3, (refer to Appendix A for formulations).

The frozen soil samples were removed from the freezer and allowed to thaw. When the samples were at room temperature 10 ml of soil were removed. The remnants were refrozen. The removed 10 ml of soil were diluted with 90 ml of sterile distilled water. Two-tenths ml of each diluted soil sample were used to inoculate plates of modified Sabouraud agar, pH4, and plates of modified Minimal Agar Davis, pH4, (refer to Appendix A for formulations).

Any visible bacterial growth was streaked on Tryptic Soy Agar (TSA) plates. Colonies from these plates were then streaked again to achieve isolated colonies. These colonies were used to inoculate TSA plates and Trypticase Soy Broth (TSB) tubes.

The following staining procedures were performed on the isolated organisms: Gram stain, acid-fast stain, and

flagellar stain.

Morphologies of the isolated colonies and individual isolates were observed and recorded.

The following biochemical tests were performed on the isolates:

- anaerobic capabilities
- antibiotic sensitivities
- catalase
- citrate
- gelatin hydrolysis
- glucose fermentation
- H<sub>2</sub>S production
- indole
- lactose fermentation
- litmus milk
- methyl red
- milk hydrolysis
- motility
- nitrate reduction
- spore production
- starch hydrolysis
- sucrose fermentation
- tributylin hydrolysis
- Voges-Proskauer

Test tubes of modified TSB, pH3, pH4 and pH5 were inoculated with each isolate. The pH of the broths was lowered by adding H<sub>2</sub>SO<sub>4</sub>. PH was tested with litmus paper.

Isolates that were able to grow at lower pH than the majority of the isolates were used to inoculate Enterotubes.

## RESULTS

Soil temperatures, pH, types and textures are listed in Table 2.

The first attempt to get bacterial growth, using modified Sabouraud Dextrose Broth, pH1, and modified Minimal Broth Davis, pH1, failed. The only growth that was noted after 5 wk of incubation was fungus. The second attempt to get bacterial growth, using modified Sabouraud agar, pH3, and modified Minimal Agar Davis, pH3, also failed. The only growth noted after 4 wk of incubation was fungus. The third attempt, however, using the modified Sabouraud agar, pH4, yielded bacterial growth. The modified Minimal Agar Davis, pH4, did not yield bacterial growth.

Eleven bacteria were isolated from the modified Sabouraud agar, pH4; two from soil sample 3, two from soil sample 6, none from soil sample 9, and one from each of the other soil samples. One of the isolates from soil sample 6, isolate 6A, was Gram-positive. The rest of the isolates were Gram-negative.

The colony and bacterial morphologies are given in Table 3. Figure 1 contains the cellular morphology of selected isolates. The results of the biochemical tests are in Table 5. The results of growth in the modified TSB, pH3, pH4 and pH5, are in Table 6. The results of the Enterotube are in Table 7.

The Gram-positive isolate, isolate 6A, was found to be

in the genus Bacillus. The Gram-negative isolates from soil sample 2, isolate 2 and from soil sample 3, isolate 3B, were found to be in the family Enterobacteriaceae. They are probably of the genus Erwinia.

Table 2. Soil Temperatures, pH's, Types, Origins, and Textures of Montanan Coniferous Bed and Field Soil Samples.

SOIL SAMPLE	TEMPT.	pH	TYPE	ORIGIN	TEXTURE
1	25 C	8.50	CHERNOZEMIC	FIELD	SANDY (FINE) AND DRY
2	25 C	8.50	CHERNOZEMIC	FIELD	SANDY (FINE) AND DRY
3	23 C	8.70	CHERNOZEMIC	FIELD	COURSE AND DRY
4	30 C	8.41	CHERNOZEMIC	FIELD	COURSE AND DRY
5	30 C	8.53	CHERNOZEMIC	FIELD	COURSE AND DRY
6	20 C	7.50	PODZOIC	CONIFEROUS BED	FINE, SMALL LUMPS
7	20 C	6.95	PODZOIC	CONIFEROUS BED	FINE, SMALL LUMPS
8	22 C	7.05	PODZOIC	CONIFEROUS BED	FINE, SMALL LUMPS
9	23 C	5.80	PODZOIC	CONIFEROUS BED	FINE, SMALL LUMPS
10	23 C	6.70	PODZOIC	CONIFEROUS BED	FINE, SMALL LUMPS

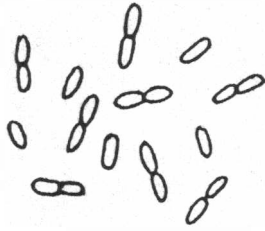
Table 3. Description of Colonial and Cellular Morphologies of Bacterial Isolates from Montanan Coniferous Bed and Field Soil Samples.

Soil Sample

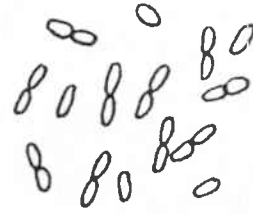
- 1 Isolate 1: Short rods (0.5  $\mu$ m x 1.60  $\mu$ m). Paired or single. Colony morphology: Small (2 mm diameter). Tannish cream color. Opaque. Glossy, round, domed. Smooth edges. Creamy consistency.
- 2 Isolate 2: Short rods (0.5  $\mu$ m x 1.60  $\mu$ m). Paired or single. Colony morphology: Small (1.5 mm diameter). Tannish color. Translucent. Glossy, round, domed. Rough edges. Buttery consistency.
- 3 Isolate 3A: Short rods (0.5  $\mu$ m x 1.2  $\mu$ m). Paired or single. Colony morphology: Same as isolate 1.
- 3 Isolate 3B: Short rods (0.5  $\mu$ m x 1.0  $\mu$ m). Paired or single. Colony morphology: Small (0.5 mm diameter). Tannish color. Translucent. Glossy, round, domed. Smooth edges. Creamy consistency.
- 4 Isolate 4: Short rods (0.5  $\mu$ m x 1.0  $\mu$ m). Paired or single. Colony morphology: Same as isolate 1.
- 5 Isolate 5: Short rods (0.5  $\mu$ m x 1.0  $\mu$ m). Paired or single. Colony morphology: Same as isolate 1.
- 6 Isolate 6A: Rods (1  $\mu$ m x 2.6  $\mu$ m). Mostly single, some paired. Colony morphology: Large (7 mm diameter). Cream color. Opaque. Glossy, round, raised dome. Smooth edges. Creamy consistency.
- 6 Isolate 6B: Short rods (0.5  $\mu$ m x 1.5  $\mu$ m). Paired or single. Colony morphology: Small (1.5 mm diameter). Tan color. Translucent. Glossy, domed. Rough undulate edges. Buttery consistency.
- 7 Isolate 7: Short rods (0.5  $\mu$ m x 1.5  $\mu$ m). Paired or single. Colony morphology: Medium (3 mm diameter). Creamish tan color. Glossy, round,

Table 3. (cont'd)

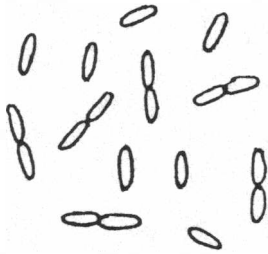
	domed. Smooth edges. Creamy consistency.
8	Isolate 8: Short rods (0.5 um x 1.5 um). Paired or single. Colony morphology: Same as isolate 7.
10	Isolate 10: Short rods (0.5 um x 1.5 um). Paired or single. Colony morphology: Same as isolate 7.



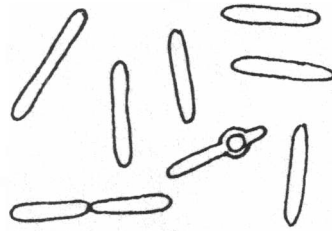
Isolate: 3B  
0.5  $\mu$  x 1.0  $\mu$



Isolate: 5  
0.5  $\mu$  x 1.0  $\mu$



Isolate: 7  
0.5  $\mu$  x 1.5  $\mu$



Isolate: 6A  
1.0  $\mu$  x 2.6  $\mu$

Figure 1. Cellular Morphologies of Selected Bacterial Isolates from Montanan Coniferous Bed and Field Soil Samples.

Table 4. Staining Reaction Results for Bacterial Isolates from Montanan Coniferous Bed and Field Soil Samples.

ISOLATE	GRAM STAIN	ACID-FAST STAIN	FLAGELLAR STAIN
1	-	-	single, polar
2	-	-	peritrichous
3A	-	-	peritrichous
3B	-	-	peritrichous
4	-	-	peritrichous
5	-	-	peritrichous
6A	+	-	single, polar
6B	-	-	peritrichous
7	-	-	peritrichous
8	-	-	peritrichous
10	-	-	peritrichous

Table 5. Biochemical Test Results for Bacterial Isolates from Montanan Coniferous Bed and Field Soil Samples.

Isolate	1	2	3A	3B	4	5	6A	6B	7	8	10
TEST											
Anaerobic	+	+	+	+	+	+	+	+	+	+	+
Antibiotic Sensitivity											
C	S	S	S	S	R	R	S	S	R	S	R
SD	S	S	R	I	S	I	S	S	S	I	S
Pen	R	R	R	R	R	R	S	R	R	R	R
E	R	R	R	R	R	R	S	R	R	R	R
NA	R	S	I	I	I	R	S	S	R	I	S
TE	I	I	I	I	S	I	S	I	S	S	S
NB	S	I	I	I	S	R	R	R	R	R	R
S	S	I	I	I	S	R	R	R	S	I	I
Catalase	+	++	+	+	+	+	+	++	+	+	+
Citrate	+	+	+	+	+	+	-	+	+	+	+
Gelatin											
Hydrolysis	+	+	+	+	+	+	-	+	+	+	+
Glucose Fermentation	+	+	+	+	+	+	+	+	+	-	-
H <sub>2</sub> S	-	-	-	-	-	-	-	-	-	-	-
Indol	-	-	-	-	-	-	-	-	-	-	-
Lactose Fermentation	-	-	-	-	-	-	+	-	-	-	-
Litmus Milk	P	P	P	P	P	P	LF	P	P	P	P
Methyl Red	-	-	-	-	-	-	+	-	-	-	-
Milk Hydrolysis	+	+	+	+	+	+	-	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reduction	-	+	-	+	-	-	+(PN)	-	-	-	-
Sporulation Production	-	-	-	-	-	-	+	-	-	-	-
Starch Hydrolysis	-	-	-	-	-	-	+	-	-	-	-
Sucrose Fermentation	-	-	-	+	-	-	+	+	-	-	-
Tributyryn Hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	+	-	-	-	-	-	-	-

+ = Positive test  
 - = Negative test  
 C = Chloramphenicol  
 SD = Sulfadiazine  
 Pen = Penicillin  
 E = Erythromycin  
 NA = Naladixic Acid  
 TE = Tetracycline

R = Resistant  
 I = Intermediate  
 S' = Sensitive

NB = Novobiocin  
 S = Streptomycin  
 PN = Past Nitrite  
 P = Proteolysis  
 LF = Lactose Fermentation

Table 6. Growth of Bacterial Isolates from Montanan Conifer-  
our Bed and Field Soil Samples in TSB, pH3, pH4  
and pH5.

Isolate	GROWTH IN THE TSB pH3, pH4, pH5		
	pH3	pH4	pH5
1	-	-	+
2	-	-	+
3A	-	-	+
3B	-	-	+
4	-	+	+
5	-	+	+
6A	-	-	-
6B	-	-	+
7	-	+	+
8	-	-	+
10	-	-	+

+ = Growth

- = No growth

Table 7. Enterotube Results for Bacteria Isolates 4, 5 and 7 from Montanan Coniferous Bed and Field Soil Samples.

Isolate	4	5	7
TEST			
Glucose Fermentation	+	+	+
Lysine Decarboxylase	-	-	-
Ornithine Decarboxylase	-	-	-
H <sub>2</sub> S Production	-	-	-
Indol Formation	-	-	-
Adonitol Fermentation	-	-	-
Lactose Fermentation	-	-	-
Arabinose Fermentation	-	-	-
Sorbitol Fermentation	-	-	-
Voges-Proskauer	-	-	-
Dulcitol Fermentation	-	-	-
Phenylalanine Deaminase	-	-	-
Urease	-	-	-
Citrate	+	+	+

## DISCUSSION AND CONCLUSIONS

The most aciduric bacteria that were isolated were those that could grow at pH4.0. This is within the pH range for most bacteria, but it is still a low pH. Since only 11 isolates were found, it could be assumed that these bacteria are more acid tolerant than other soil bacteria in the soil samples.

The reason results of growth in TSB, pH3, pH4 and pH5 do not correlate with results of growth on Sabouraud agar pH4 is because the preparation of the agar included a dilution that raised the pH of the agar. This dilution, caused by the addition of hydrated agar-agar into the acidified Sabouraud agar, will raise the pH about 0.5. Therefore, the isolates may have initially been isolated in an environment of pH 4.5 rather than pH4.

All but one of the isolates were Gram-negative, rod-shaped, facultative anaerobes. This was expected because most soil bacteria are Gram-negative, rod-shaped, facultative anaerobes (4). The Gram-positive rod was probably isolated because it is a spore former, and when it was transferred to normal pH TSA plates, the bacteria could grow well. One good aspect about isolation of this Gram-positive rod was that it served as a good reference for most of the biochemical tests. Usually, the Gram-positive rod had opposite results compared to the Gram-negative rods.

There were four different Gram-negative isolates from the coniferous bed soil and six different Gram-negative isolates from the field soil. More bacteria from the coniferous bed soil might be expected because coniferous bed soil is more acidic than the field soil. Since the field soil environment is usually more rich than the coniferous bed soil, there may be a greater diversity of bacteria and, hence, more that can live in a low pH environment.

The tests used reveal that all the bacteria isolated are different, since none of them have the exact same test results. The Gram-bacteria have some of the same test results, i.e., all are motile, all are facultative anaerobes, all can use citrate as their sole carbon source, and all can produce catalase. There are tests that separate the organisms, namely the antibiotic sensitivities, glucose fermentation, nitrate reduction, sucrose fermentation and Voges-Proskauer.

The enterotube had six tests that were the same as the biochemical tests that were prepared and performed separately. These tests are glucose fermentation, H<sub>2</sub>S production, indol formation, lactose fermentation, Voges-Proskauer, and citrate. The Enterotube results for isolates 4,5, and 7 are the same as the results from the separately performed tests. This suggests that the tests were performed correctly.

The biochemical tests were performed because they are some of the most common tests used to identify bacteria.

The H<sub>2</sub>SO<sub>4</sub> was used to lower the pH of the modified media because it is diprotic. It, therefore, requires less H<sub>2</sub>SO<sub>4</sub> to lower the pH. Also, sulfuric acid is a source of sulfate.

Complete identification of the isolates was not achieved. Except for two of the isolates isolated, no family identification was made. Either a main biochemical characteristic was different, or too many minor biochemical characteristics were different. Difficulty with some of the identifications of Gram-negative, facultative anaerobic, acid tolerant bacilli from soil suggest much more work is desired on the taxonomy of these organisms.

Two bacteria were classified in the family Enterobacteriaceae because they are motile by flagella, not acid-fast, facultatively anaerobic, and catalase positive. Mainly, though, they have the ability to reduce nitrates to nitrites. They are thought to belong to the genus Erwinia.

If this project were to be continued, it is suggested to use a wider variety of media. It should be noted that one of the two media used produced growth. Perhaps a better anti-fungal agent may be incorporated into the media to stop fungal growth that may become inhibitory to desired bacterial growth.

In conclusion, different isolates that could grow in a low pH were isolated from Montanan coniferous bed and field soil samples. Some of the isolates' properties and

characteristics were determined. No complete identification was achieved, but some partial identification was accomplished.

APPENDIX A

SABOURAUD AGAR pH3 or pH4

Ingredients	Amount
Neopeptone, Difco	10 g.
Bacto-Dextrose	40 g.
Agar-Agar	15 g.
Distilled Water	600 ml.
H2S04	X ml.
Agar-Agar	30 g.
Distilled Water	400 ml.
Fungizone (Amphotevicin B)	10 ml. @ 250 mg/ml

MINIMAL AGAR DAVIS pH3 or pH4

Ingredients	Amount
Bacto-Dextrose	1.0 g.
Dipotassium Phosphate	7 g.
Monopotassium Phosphate	2 g.
Sodium Citrate	0.5 g.
Magnesium Sulfate	0.1 g.
Ammonium Sulfate	1.0 g.
Distilled Water	600 ml.
H2S04	X ml.
Agar-Agar	30 g.
Distilled Water	400 ml.
Fungizone (Amphotevicin B)	10 ml. @ 250 mg/ml

For the above media:

The first set of ingredients were added into the 600 ml of distilled water. H2S04 was added to this solution to lower the pH to a pH slightly lower than the desired pH. This was done to account for the dilution factor when the agar-agar solution was added to it. The pH was tested with litmus paper. The solution was heated to a boil for 1 to 2 min, then poured into bottles and capped. The agar-agar was added to the 400 ml of distilled water, then heated to a boil for 1 to 2 min. This solution was poured into a bottle, then capped. The two solutions were autoclaved at 121 C at 15 psi for 15 minutes. After the two solutions cooled to about 50 C, the agar-agar solution was poured in- to the liter bottle the nutrient solution was in. The

APPENDIX A (cont'd)

Fungizone was added to this mixed solution. This total solution was then poured into plastic petri dishes and allowed to cool. The extra agar is added because the H<sub>2</sub>SO<sub>4</sub> destroys the agar in the autoclaving process, and without the agar, solidification does not occur. The Fungizone is added as an anti-fungal agent, to reduce fungal contamination.

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