STUDIES ON THE GENUS MYCOPLANA

by

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TABLE OF CONTENTS

INTRODUCTION

The ability of microorganisms to utilize the benzene ring as a sole source of carbon has been recognized since the early part of this century. This type of metabolism is rather unique but exists in several unrelated groups of microorganisms.

The genus Mycoplana was originally described as a new genus by Gray and Thornton (1928). At that time, the taxonomic relationship to other known forms of bacteria was considerably in doubt. The descriptions presented were not sufficiently conclusive to establish it as a member of the family Pseudomonadaceae, however, the genus was placed in this family more or less tentatively by the editors of Bergey's Manual. Mycoplana was given generic status by virtue of branching cells and the ability to metabolize phenol. The branching of the cells differentiated Mycoplana from certain members of the genus Pseudomonas since many species of Pseudomonas share similar biochemical abilities with Mycoplana, among these being the utilization of certain aromatic compounds. It would seem that a closer relationship exists between the two genera than is indicated by the presently accepted taxonomic relationship.

Little reference is made in the literature to the genus Mycoplana, since the genus is of no apparent economic or pathologic importance.

This study was undertaken with the desire to obtain a fuller knowledge of the physiologic activities of the group and to help

clarify the taxonomic position of the genus in regard to other known species of microorganisms.

REVIEW OF LITERATURE

The ability of bacteria to decompose the benzene ring was first reported by Störmer (1908). He stated that in testing soil with various organic compounds including toluene, xylene, and cresol, he obtained microorganisms that could decompose phenol and cresol, however, details of this phase of the work were not given.

The first conclusive evidence that certain aromatic compounds could serve as sole carbon and energy sources for various soil bacteria was given in 1914 by Wagner. Using seven species of bacteria isolated from dust, soil, and various animal excrements, he showed that phenol and phloroglucinol were oxidized to carbon dioxide, pyrocatecol to hydroquinone, and benzene to fatty acids and carbon dioxide. Toluene, xylene, and guaicol were decomposed. Alkaloids and terpenes, with the exception of menthol, were not attacked.

In 1915 Buddin tested various antiseptic agents with relation to their effect on the bacterial population of soil and production of ammonia and nitrates therein. He found that the compounds tested could be generally placed into two groups. The first group was composed of inorganic compounds and volatile substances (e.g., toluene) which would rapidly disappear from the

soil by virtue of volatility. This group of compounds produced an initial decrease followed by a big increase in the soil population, and ammonia production was sustained for a long period of time. The second group of compounds consisted of organic substances that were, as a rule, less volatile; among this group were phenol, the cresols, and naphthalene. They produced an effect that differed markedly from that of the first group in that upon their addition to the soil a large increase in bacterial population ensued but decreased rapidly. No significant increase followed the population rise, indicating that energy was not derived from organic nitrogen. It was suggested that the antiseptics were being utilized by the bacteria.

An observation similar to Buddin's was made by Sen Gupta (1921). His work suggested that the disappearance of phenol and cresol, when added to the soil, was due to the presence of a biological agent since no disappearance occurred when the soil was sterilized.

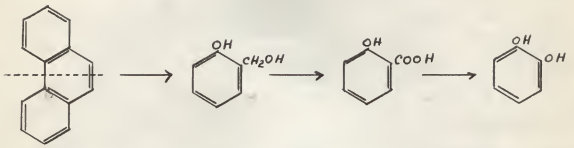
The lines of research begun by Buddin were further extended by Matthews (1924) to a large number of organic compounds and her findings gave further support to the hypothesis that many organic antiseptics can be used as a food supply by some of the soil bacteria. Her work showed a relationship existing between growth and the heat of combustion and molecular weight of the compound used.

Up to 1928 no mention was made of the identity or taxonomic relationships of microorganisms having the ability to cleave the benzene ring. Gray and Thornton (1928) isolated from the soil a

number of organisms having this ability. They were placed into seven genera comprising twenty-five species. One of these genera was the genus Mycoplana which Gray and Thornton described as a new genus. In the initial classification of Mycoplana, the genus was placed in the family Mycobacteriaceae Chester. The authors stated that the classification used by them was not that of a permanent type but rather one of convenience. The genus was later moved to the family Pseudomonadaceae Winslow et al. This change is justified because although Gray and Thornton described Mycoplana as having branching cells, the fact remains that the cells are polar flagellated and non acid fast. Included among the other genera that could use the benzene ring were members of Micrococcus, Mycobacterium, Bacterium, Pseudomonas, Vibrio, and Bacillus.

Several investigators have concerned themselves specifically with the mechanisms of the attack upon the benzene ring. Tausson (1929) investigated the oxidation of certain condensed ring aromatic hydrocarbon compounds and drew deductions concerning possible pathways of breakdown. He used the naphthalene decomposing organism Bacterium naphthalinicum, and found that naphthols, diphenols, triphenols, and phthalic acids were not attacked. He considered these substances to be the only likely products of an initial oxidation of one of the two rings of naphthalene, hence he concluded that the attack involved simultaneous rupture of the two condensed nuclei of the naphthalene ring. Tausson, however could not obtain actual evidence of his postulation. The organism

attacking phenanthrene grew well only on o-hydroxybenzyl alcohol, salicylic acid, and catechol. This suggests a direct relationship between the oxidation of the four substances. Tausson postulated the following scheme:

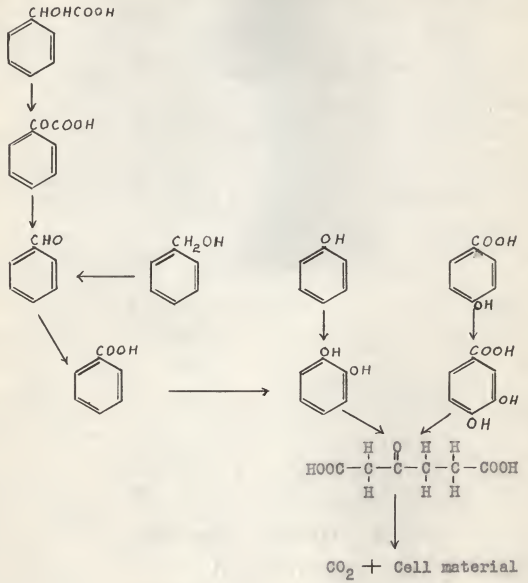


Bernheim (1942) used manometric techniques to investigate the decomposition of benzoic acid and related compounds by Mycobacterium spp. He concluded that the decomposition of benzoic acid was via one of the isomeric monohydroxy benzoic acids.

Evans (1947), when using the aerobic bacterium Vibrio 01, obtained strong evidence for the production of 3,4 dihydroxybenzoic acid from m- and p- hydroxy benzoic acid when the latter compounds were used as sole carbon sources.

The most extensive work on the mechanism of the benzene ring cleavage has been done by Stanier (1948), Sleeper and Stanier (1950), and Stanier et al. (1950). The most advanced conception (Stanier et al. 1950) is the mechanism involved when catechol and protocatechuic acid are oxidized by Pseudomonas fluorescens. By the use of manometric methods, adaption studies, and enzymatically active cells, Stanier and his co-workers found that beta-ketoadipic acid was a common intermediary metabolite of both catechol

and protocatechuic acid and was also to be found intermediary in the oxidation of many other aromatic substances by Pseudomonas fluorscens. They have proposed the following reaction scheme:



They admit, however, that the detailed mechanism for the transformation of catechol and protocatechuic acid to beta-ketoadipic acid is unknown.

The only other reference found to the genus Mycoplana was that made by Bushnell and Haas (1941) who tested it, along with several other unrelated genera, for the ability to utilize kerosene as a sole source of carbon.

Experiment 1: ISOLATION AND SELECTION OF CULTURES EMPLOYED

The original source of Mycoplana bullata and M. dimorpha was from garden plot soil at the Rothamsted Experiment Station, Harpenden, England. This knowledge, plus the fact that only three strains, one of M. dimorpha and two of M. bullata were isolated and described (Gray and Thornton, 1928), gave considerable speculation as to the extent of distribution in this country. It was felt, however, that due to the ubiquitous nature of soil organisms, the occurrence of the Mycoplana species in Kansas was highly possible.

Gray and Thornton reported three methods for the isolation of microorganisms having the ability to utilize phenol and the cresols. The first method consisted of the addition of 50 mgm of phenol or m-cresol to 100 gms of soil. At the conclusion of the incubation period, platings were made from a diluted suspension of this soil onto a nutrient agar medium. Representative colonies were picked and tested for their ability to grow on a mineral salt substrate with phenol or m-cresol as the sole source of energy. The second method differed from the first in that approximately one gram of soil was initially inoculated

into 100 ml of a solution of mineral salts to which had been added 0.05 percent to 0.1 percent phenol or 0.05 percent m-cresol. If growth occurred, transplants were made into flasks of the same medium from which subsequent platings were made as described above, the predominant organism was isolated and inoculated into phenol or m-cresol media. The third method described was the plating of soil suspensions onto an agar medium made up with mineral salts solution to which phenol or m-cresol was added. Gray and Thornton stated that of the three methods used, the second technique was most successful.

Organisms used in this study were isolated by a combination of methods two and three as stated above. It was felt that the combination gave more selectivity and ultimately decreased the time required to obtain a flora of the ring decomposing group.

The composition of the medium employed in the initial isolations was as follows:

K_2HPO_4	1.0 gm
$MgSO_4.7H_2O$	0.2 gm
NaCl	0.1 gm
$CaCl_2.2H_2O$	0.1 gm
$FeCl_3.6H_2O$	0.01 gm
$(NH_4)_2SO_4$	1.0 gm
Distilled water	1000 ml

The medium was adjusted to pH 7.2 to 7.3 with N/1 NaOH using brom thymol blue as an indicator. The medium was dispensed in 100 ml amounts and sterilized at 15 pounds pressure for 20 minutes. The solid mineral salts contained, in addition, 1.5

percent of agar-agar. Just prior to inoculations, 0.1 ml of 90 percent phenol was added thus giving a final phenol concentration of 0.09 percent.

One hundred and twenty-four soil samples were obtained from nine eastern counties of Kansas and from one county in western Missouri. Field and garden soils predominated the selection, however, nine samples were taken from sources which had been exposed to previous contact with hydrocarbon compounds of a phenolic nature; i.e., creosote and cresol compounds used as a wood preservative and as an agent for the removal of hair from slaughtered animals.

The samples were gathered in approximately one gram amounts and were transported to the laboratory in sterile vials. The moisture level was not altered mechanically in any way. Inoculations into the mineral salts solution were completed before an elapse of a maximum of 12 hours. The containers used for incubation were standard six ounce, screw-cap bottles. The caps were loosely attached thus permitting passage of air into the container.

The incubation period of the primary enrichment cultures was seven days, and room temperature (approximately 22°C.) was used as the temperature of incubation. A certain amount of temperature fluctuation could be expected, but the flasks were stored in a cupboard free from any undue amounts of circulating air currents, thus, holding the variation to a minimum.

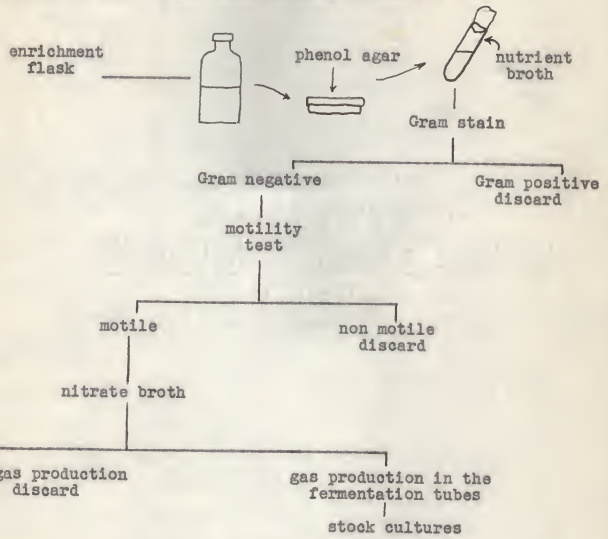
At the conclusion of the incubation period, the flasks were

agitated to permit thorough mixing and were allowed to stand to effect the settling out of the particulate matter. A single loopful of the supernatant liquid was then streaked onto mineral-salts phenol agar plates having a concentration of 0.09 percent phenol. The plates were incubated at room temperature for five to seven days, the usual optimum being seven days for adequate growth suitable for pure culture isolation.

From the mineral-salts phenol agar plates, colonies resembling the description recorded by Gray and Thornton were picked into tubes of nutrient broth and incubation at 30°C. for 24 hours. Gram stains were made of the broth cultures following Hucker's method as outlined in the Manual of Methods for Pure Culture Study of Bacteria. All Gram positive cultures were discarded at this point. All Gram negative cultures were observed for motility with all non-motile organisms being discarded.

The remaining Gram negative motile organisms were inoculated into nutrient broth containing 0.1 percent KNO_3 as a readily available nitrate source. A small fermentation vial was included in each tube of the nitrate broth. These tubes were allowed to incubate at 30°C. for 24 hours. The cultures were read for the presence or absence of gas in the inverted fermentation vial. The cultures which produced gas in the nitrate broth were retained as the working stock cultures while those which showed absence of gas production were discarded. This criterion was used since in the original description, M. bullata and M. dimorpha were the only organisms isolated from the soil by Gray

Thornton with the ability to split the benzene ring and also able to reduce the nitrate radical to free nitrogen. Graphically one can represent the isolation technique as follows:



By the use of the isolation technique described, a total of 15 cultures was selected for further taxonomic study. Type cultures of Mycoplana bullata (ATCC 4278) and M. dimorpha (ATCC 4279) were obtained from the American Type Culture Collection and were used as reference cultures.

Experiment 2: DETAILED MORPHOLOGIC STUDY OF *MYCOPLANA* SPECIES

In the original description of the *Mycoplana* species by Cray and Thornton, the statements regarding flagellation are contradictory. *Mycoplana bullata* is described as having flagella either polar or peritrichous; *M. dimorpha* having flagella both polar and peritrichous. The drawings given in the original citation usually indicated peritrichous rather than polar flagellation. If these organisms exhibited peritrichous flagellation, then placement in the family *Pseudomonadaceae* is incorrect.

Flagella stains were prepared of the soil isolates and of both reference cultures. The technique used to demonstrate flagellation was the Plimmer and Paine modification of Casares-Gil's flagella stain. Strict adherance was maintained to the recommended procedures as outlines in the Manual of Methods for Pure Culture Study of Bacteria.

Upon microscopic examination of the prepared flagella stains, both species of *Mycoplana* and all of the soil isolates were observed to possess polar flagellation. The number of polar flagella varied from one to several, however monotrichous forms predominated. Figures 1 and 2 show flagellation typical for *Mycoplana dimorpha* and *M. bullata*.

Fig.1. Cells of M. bullata showing monotrichous flagellation. x 1000.

Fig.2. Cells of M. dimorpha showing monotrichous flagellation. x 1000.

Experiment 3: TOLERANCE OF PHENOL BY MYCOPLANA SPECIES

The ability of certain microorganisms to metabolize phenol is remarkable because phenol is a very active bactericide if concentration is sufficient. Most bacteria are killed in 5 to 10 minutes by a dilution of 1:80 or 1:110 at room temperature, with bacterial spores being more resistant. The ability of this group of organisms to metabolize phenol is dependent upon the concentration. The level of concentration must not be that where toxicity occurs, otherwise metabolic activity will be suppressed, yet must be sufficient to support growth.

Suspensions of the soil isolates and of the reference cultures were streaked onto plates of mineral salts agar having phenol added in the following concentrations: 0.05 percent, 0.10 percent, 0.15 percent, and 0.20 percent. Incubation was maintained at 30°C. with observation being made at 24 hour intervals.

Good growth was obtained on the plates containing 0.05 percent phenol, while best growth was obtained on the 0.1 percent series. Only slight growth occurred, if at all, on the plates containing 0.15 percent phenol, while no growth occurred on the 0.2 percent phenol plates (seven days incubation).

The ability to tolerate phenol in the presence of organic matter was tested by means of tubes of nutrient broth having progressively increasing amounts of phenol added. Table 1 presents results obtained when test organisms were grown in the presence of varied amounts of phenol.

Table 1. Ability of reference cultures and soil isolates to tolerate phenol in the presence of organic matter.

Organism	Concentration of phenol 1 part in											
	450	500	550	600	650	700	750	800	850	900	950	1000
ATCC 4278	-*	-	-	-	-	X	X	X	X	X	X	X
ATCC 4279	-	-	-	-	X	X	X	X	X	X	X	X
41-2	-	-	-	-	-	-	-	-	-	-	-	X
53-1A	-	-	-	-	X	X	X	X	X	X	X	X
53-1B	-	-	-	-	X	X	X	X	X	X	X	X
53-1C	-	-	-	-	X	X	X	X	X	X	X	X
53-1D	-	-	-	-	X	X	X	X	X	X	X	X
53-2	-	-	-	-	X	X	X	X	X	X	X	X
91-1	-	-	-	-	X	X	X	X	X	X	X	X
91-4	-	-	-	-	X	X	X	X	X	X	X	X
106-1	-	-	-	-	X	X	X	X	X	X	X	X
106-2	-	-	-	-	X	X	X	X	X	X	X	X
106-5	-	-	-	-	X	X	X	X	X	X	X	X
110-1	-	-	-	-	X	X	X	X	X	X	X	X
110-2	-	X	X	X	X	X	X	X	X	X	X	X
110-3	-	-	-	X	X	X	X	X	X	X	X	X

* -, no growth; X growth. All incubations were at 30°C. for 24 hours.

Experiment 4: REDUCTION OF THE NITRATE RADICAL BY MYCOPLANA SPECIES

In the original description of Mycoplana bullata and M. dimorpha by Gray and Thornton (1928), the production of nitrites from potassium nitrate was recorded as negative. They noted that gas, presumably free nitrogen, was produced from the nitrate broth. The results obtained in this study substantiate in part the results as originally described.

It was observed that both species of Mycoplana were distinctly nitrite positive in 24 hours thus differing from the results obtained by Gray and Thornton. However, the method used by the original authors can be criticized. They used a seven day incubation period before testing for nitrites, and that elapse of time could account for the absence of the nitrite intermediate.

The medium used for the demonstration of nitrite production was standard nutrient broth with 0.1 percent KNO_3 added. Small fermentation vials were added prior to sterilization of the medium. Sterilization was done at 15 pounds steam pressure for 20 minutes. All incubations were maintained at 30°C.

Three series of tubes were set up, one inoculated with Mycoplana bullata (ATCC 4278), one with M. dimorpha (ATCC 4279), and the last maintained as an uninoculated control. The control was run to guard against error from nitrate reduction due to medium components and from possible absorption of nitrous acid from the air.

The method of testing for the presence of nitrites was the

alpha naphthylamine-sulphanilic acid technique; a few drops of each reagent being added to the culture tube allowing sufficient time for color development. Table 2 shows the relationship of the presence of nitrites to the hours of incubation.

Table 2. Relationship of nitrite production to hours of incubation by Mycoplana reference species.

Series	Hours of incubation at 30°C.					
	24	48	72	96	120	144
ATCC 4278	XX*	XX	XX	X	X	-
ATCC 4279	XX	XX	XX	X	X	-
Control	-	-	-	-	-	-

* XX, strong nitrite reaction; X, weak nitrite reaction; -, negative nitrite reaction. Gas production by the reference cultures occurred 24 hours after incubation.

This experiment shows that in the reduction of potassium nitrate to free nitrogen, a nitrite intermediate can be strongly detected for 72 hours after inoculation, while negative results were obtained after 144 hours (six days) incubation. It is evident, therefore, that Gray and Thornton carried out their tests after decomposition had proceeded beyond the nitrite stage.

Table 3 presents the results obtained when the soil isolates were tested for the presence of nitrites in relationship to hours of incubation.

Table 3. Relationship of nitrite production to hours of incubation by the soil isolates.

Series	Hours of incubation at 30°C.					
	24	48	72	96	120	144
41-2	X*	-	-	-	-	-
53-1A	X	-	-	-	-	-
53-1B	X	-	-	-	-	-
53-1C	X	-	-	-	-	-
53-1D	X	-	-	-	-	-
53-2	XX	X	-	-	-	-
91-1	X	-	-	-	-	-
91-4	X	-	-	-	-	-
106-1	-	-	-	-	-	-
106-2	-	-	-	-	-	-
106-5	X	-	-	-	-	-
106-6	X	-	-	-	-	-
110-1	X	-	-	-	-	-
110-2	XX	XX	XX	X	X	-
110-3	XX	XX	XX	X	X	-

* XX, strong nitrite reaction; X, weak nitrite reaction; -, negative nitrite reaction. Gas production obtained in 24 hours except culture 106-1, 106-2, 110-1, 110-2, and 110-3. The production of gas was regained in cultures 110-2 and 110-3 upon passage through nitrite broth.

It is to be noted that cultures 106-1 and 106-2 have lost the ability to produce free nitrogen in the fermentation vials. The presence of nitrites could not be detected in either 0.1 percent or 1.0 percent KNO_3 broth. Passage through nitrate broth (two weeks) did not restore these abilities. Culture 110-1 did not regain the ability to reduce nitrates to nitrites upon passage through nitrate broth. These cultures produced gas in the fermentation vials and reduced 0.1 percent KNO_3 broth upon initial isolation.

Experiment 5: EXPERIMENTS TO INDUCE BRANCHING OF CELLS OF MYCOPLANA SPECIES

The separation of the genus Mycoplana from the other genera of the family Pseudomonadaceae is on the morphologic characteristic of branching cells. Camera lucinda drawings presented in the original citation by Gray and Thornton (1928) show that the cells are, for the most part, "Y" shaped. Gray and Thornton state that the branching of the cells occurs especially in young cultures.

It was noted early in these studies that no evidence of branching could be detected upon microscopic observation, and since this characteristic is paramount as a taxonomic feature, the importance of branching becomes readily apparent.

The photomicrographs shown in Figures 3 and 4 were made of young cells (18 hours old) grown upon nutrient agar and are

stained with Rucker's crystal violet. It is felt that a dye such as crystal violet gives only minimal distortion to the cells.

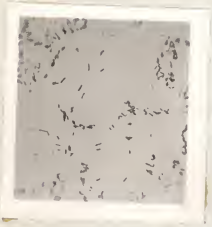


Fig. 3. Young cells of M. bulleta grown on nutrient agar. x 1000.

Fig. 4. Young cells of M. dimorpha grown on nutrient agar. x 1000.

Several methods were employed in an attempt to induce branching of the reference cultures and soil isolates. The methods were designed to bring about unfavorable cultural and environmental conditions.

The effect of variation in pH was determined by means of tubes of nutrient broth which were adjusted from pH 4 to pH 7. The adjustments were made with 1/N HCL using electrometric technique. For pH 8 and pH 9, adjustments were made with 1/N NaOH. The adjustments were made prior to sterilization. After inoculation, all incubations were for 24 hours at 30°C.

To ascertain the effect of temperature of incubation, three series of nutrient agar slants were inoculated with test organisms. These slants were then placed at 15°C., 30°C., and 37°C.,

respectively. At the conclusion of the incubation period (24 hours), stains were made of the cultures. These cultures were incubated further for a total of 7 days thus introducing age as an additional factor and at which time additional stains were made.

Colonies of the test organisms were picked directly from the mineral salts phenol agar plates used in the utilization studies. These cells were stained directly to determine what effect phenol would have upon cell morphology. The age of the cells was, in this case, 72 hours.

The effect of reduced surface tension was studied by the use of nutrient agar with 0.1 percent sodium rincinoleate (Eastman Kodak Company). Stains of the test organisms grown on this medium were made at 24 hour and 72 hour incubation intervals. Incubations were maintained at 30°C.

The soil isolates and reference cultures were inoculated into deep tubes of Difco thioglycollate agar. These tubes give a gradation of oxygen tension varying from an aerobic to strictly anaerobic environments, depending upon which level the cells were removed from. It was noted, however, that no growth occurred in the deeper levels of the thioglycollate medium. The organisms growing under microaerophilic conditions were removed with Pasteur pipettes. Stains were made of the organisms after 24 hour incubation (30°C.).

The possibility of morphologic change due to varied amounts of sodium chloride was determined by inoculations into tubes of

nutrient broth containing 1.0, 1.5, 2.0, and 2.5 percent sodium chloride. Incubation was maintained at 30°C. for 24 hours before staining.

To determine the effect of dye upon the cell morphology, tubes of Difco crystal violet agar were inoculated with the test organisms. Incubation was at 30°C. for 24 hours before staining.

The ability of lithium chloride to produce aberrant morphology has been used in several studies in the past (Hadley, Delves, and Klinek, 1931) (Rettger and Gillespie, 1933). The effect of this salt upon the soil isolates and reference cultures was determined by means of a series of tubes containing 5 ml of nutrient agar with 10 percent lithium chloride added in 1 ml, 2 ml, 3 ml, and 4 ml amounts. These tubes were incubated for 48 hours at 30°C. Good growth was obtained upon the tubes containing 1 ml and 2 ml of the lithium salt.

The effect of osmotic pressure was determined by means of nutrient broth having glucose added in varied amounts. The concentration of glucose used was 4, 8, 16, 32, and 64 percent. Growth was inhibited in the higher concentrations while growth occurred in the series containing 4 and 8 percent glucose. Sterilization of the tubes was at 15 pounds steam pressure for 20 minutes. Incubation of the tubes was at 30°C. for 24 hours.

The staining technique used to demonstrate the possible variation from normal morphology was the same in all cases. The cells were stained with Hucker's crystal violet for 1 minute and then washed with distilled water.

All of the methods used in this set of experiments failed to yield any evidence of branching as was originally described by Gray and Thornton.

Figures 5 and 6 show photomicrographs of cells grown upon lithium chloride agar.

Fig. 5. Cells of M. bullata grown on lithium chloride agar. x 1000.

Fig. 6. Cells of M. dimorpha grown on lithium chloride agar. x 1000.

Experiment 6: BIOCHEMICAL CHARACTERISTICS OF MYCOPLANA SPECIES

The techniques of the biochemical tests used in this study were those recommended by the Manual of Methods for Pure Culture Study of Bacteria, however, special mention will be made concerning several of these tests.

The ability to liquify gelatin was determined by the Smith modification of the Frazier technique. This method was supplemented by the use of tubes of nutrient gelatin which were

inoculated and then incubated at 30°C. At periodical intervals, the tubes were placed at refrigeration temperature to determine whether or not the gelatin was still capable of solidifying.

The basal medium used for the sugar fermentation studies was beef extract peptone broth containing 1.0 percent of the appropriate carbohydrate. The indicator for the detection of acid production was brom cresol purple; 1 ml of a 1.6 percent alcoholic solution being added per liter of the basal medium. The pH of the basal medium was adjusted to 7.0 to 7.2 with 1/N NaOH using brom thymol blue as an indicator, the pH adjustment being made prior to the addition of the internal indicator. Sterilization of the carbohydrate medium was at 15 pounds steam for 15 minutes. Uninoculated controls were maintained as a check upon sterility of the sterilized medium.

The medium used to test for the production of hydrogen sulfide was Difco peptone iron agar. To test the efficiency of this medium, a known culture of Proteus vulgaris was used as a control. The tubes were incubated at 30°C. for seven days before discarding as negative.

The ability to hydrolyze starch was tested by the method described in the Manual of Methods for Pure Culture Study of Bacteria. Sterile Difco starch agar was poured into Petri dishes and after hardening, single streak inoculations were made up on the surface. Incubation was at 30°C. for seven days. At the termination of the incubation period, the surface of the Petri dishes were flooded with Lugol's iodine. The breath of the clear

zone outside of the area of growth indicates the extent of starch destruction.

The medium used for the production of indole was Bacto tryptone broth. Incubations were at 30°C. for 24 and 48 hours. At the end of the incubation period a small amount of xylol was added to each culture tube. The mixture was shaken vigorously and allowed to stand for a few minutes. About 1 ml of para-dimethylaminobenzaldehyde was added to the tubes but not shaken, and the tubes were allowed to stand. If indole was produced, the compound would be concentrated in the xylol layer and color development would be much more evident.

Table 4 presents a tabulation of the observed biochemical characteristics of the reference cultures and the soil isolates.

Table 4. Biochemical characteristic of the reference cultures and soil isolates.

Medium or Test	Organism																	
	4278	4279	41-2	53-1A	53-1B	53-1C	53-1D	53-2	91-1	91-4	106-1	106-2	106-5	106-6	110-1	110-2	110-3
Lactose	-*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Celliobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-
Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-
Levulose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	X	X	X	X	X	X	X	X	-	-	-	-	X	X	X	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	X	X	X	X	X	X	X	X	-	-	-	-	X	X	X	-	-

Table 4 (cont.)

Medium or Test	Organism																
	4278	4279	41-2	53-1A	53-1B	53-1C	53-1D	53-2	91-1	91-4	106-1	106-2	106-5	106-6	110-1	110-2	110-3
Catalase	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Koser's citrate	X	X	X	X	X	X	X	X	-	-	-	-	X	X	X	X	X
Indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrites	X	X	X	X	X	X	X	X	X	X	-**	-**	X	X	-**	X	X
Starch	X	X	X	X	X	X	X	X	-	-	-	-	X	X	X	X	X
Gelatin	-	-	-	-	-	-	-	-	X	X	X	X	-	-	-	X	X
Hydrogen sulfide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4 (Concl.)

Medium or Test	Organism																
	4278	4279	41-2	53-1A	53-1B	53-1C	53-1D	53-2	91-1	91-4	106-1	106-2	106-5	106-6	110-1	110-2	110-3
Action on litmus milk																	
a. peptonized	-	-	-	-	-	-	-	-	X	-	X	X	-	-	X	X	X
b. acid reduced	-	-	X	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c. alkaline reduction	X	X	-	X	X	X	X	X	-	X	-	-	X	X	-	-	-
Chromogenesis																	
a. yellow	-	-	-	-	-	-	-	-	-	-	-	-	-	-	X	X	X
b. buff	X	X	X	X	X	X	X	X	-	-	X	X	X	X	-	-	-
c. white	-	-	-	-	-	-	-	-	X	X	-	-	-	-	-	-	-

* -, no acid or gas in the case of sugar fermentations. Negative reaction in the case of other biochemical tests; X, acid, no gas in the sugar fermentations. Positive reaction in the other biochemical tests; S, slight reaction; -, no reaction or negative. ** Nitrite positive upon original isolation.

Experiment 7: UTILIZATION OF AROMATIC COMPOUNDS BY MYCOPLANA SPECIES

Although Gray and Thornton originally reported phenol as being utilized as a sole carbon and energy source by Mycoplana bullata and M. dimorpha, their studies did not indicate to what extent the Mycoplana species could metabolize other simple aromatic compounds.

The ability to utilize simple aromatic compounds was determined by growth experiments using a mineral salts agar base having the following composition:

NH_4NO_3	1.0 gm
K_2HPO_4	1.0 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
Agar-agar	20.0 gm
Distilled water	1000 mls

pH was adjusted to 7.0 to 7.2 with 1/N NaOH using brom thymol blue as an indicator. Sterilization was carried out at 15 pounds steam pressure for 20 minutes.

The mineral salts agar base was supplemented with a single specific aromatic compound to serve as the sole carbon and energy source for the test organisms. The aromatic compounds used throughout these utilization studies were obtained from the Eastman Kodak Company, Rochester, New York. Control plates containing only the mineral salts agar were included since the impurities in the agar-agar itself will sustain a small amount of growth. The usual

concentration of the aromatic compounds tested was 0.1 percent, this being sufficient for growth if the compound is metabolized. If no growth occurred, the concentration was reduced to 0.05 percent to minimize the possibility of toxicity on the part of the compound being tested.

The compounds tested were those simple benzene derivatives with no more than one carbon atom in the substuted group or groups. This minimizes the possibility of the carbon atoms of an extended side chain being utilized in preference to the carbon atoms of the benzene ring.

Suspensions were made of the soil isolates and of the reference cultures using sterile doubly distilled water. A loopful of the cell suspension was streaked onto the surface of the mineral salts aromatic substrate agar. The plates were incubated at 30°C. for 72 hours with observations made at 24 hour intervals. If utilization of the aromatic compound occurred, colonial growth would be markedly more abundant than that of the control plates.

The results recorded in Table 5 indicate the ability of the soil isolates and reference cultures to utilize mono-, di-, and tri-hydroxy phenols.

Table 5. Utilization of mono-, di-, and tri- hydroxy phenols by the reference cultures and soil isolates.

Organism	Phenol	Catechol	Resorcinol	Hydroxyquinone	Pyrogallic acid	Phloroglucinol
ATCC 4278	XXXX*	-	XXX	-	-	XXXX
ATCC 4279	XXXX	-	XXX	-	-	XXXX
41-2	XXXX	-	XX	-	-	XXX
53-1A	XXXX	-	XX	-	-	XXX
53-1B	XXXX	-	XX	-	-	XXX
53-1C	XXXX	-	XX	-	-	XXX
53-1D	XXXX	-	XX	-	-	XXX
53-2	XXXX	-	XX	-	-	XXX
91-1	X	-	X	-	-	X
91-4	XXXX	-	X	-	-	XXX
106-1	XXXX	-	X	-	-	XXX
106-2	XX	-	X	-	-	XX
106-5	XXX	-	X	-	-	XX
106-6	XXX	-	X	-	-	XX
110-1	XXXX	X	X	-	-	XXX
110-2	XXXX	-	X	-	-	XX
110-3	XXXX	-	X	-	-	XXX

* X, slight growth; XX, moderate growth; XXX, good growth; XXXX, excellent growth; -, no growth or equal to control.

Table 6 shows the results of utilization by the test organisms when grown upon toluene and substituted derivatives.

Table 6. Utilization of toluene and substituted derivatives by Myconlana species and soil isolates.

Organism	Toluene	Ortho-hydroxy toluene	Meta-hydroxy toluene	Para-hydroxy toluene	3,5 di-hydroxy toluene
ATCC 4278	-*	XXX	-	-	XXX
ATCC 4279	-	XXX	-	-	XXX
41-2	-	XXX	X	X	XXX
53-1A	-	XXX	-	X	XXX
53-1B	-	XXX	-	X	XX
53-1C	-	XXX	-	X	XX
53-1D	-	XXX	-	X	XX
53-2	-	XX	-	X	XX
91-1	-	X	-	-	X
91-4	-	X	-	XXX	XXX
106-1	-	-	-	-	XX
106-2	-	X	-	-	XX
106-5	-	XX	-	-	X
106-6	-	XX	-	-	XX
110-1	-	X	XX	XX	X
110-2	-	XX	X	X	X
110-3	-	XX	X	X	X

* X, slight growth; XX, moderate growth; XXX, good growth; XXX, excellent growth; -, no growth or equal to control.

Table 7 presents results obtained when benzoic acid and monohydroxy substituted benzoic acids were used as the aromatic substrate. Benzaldehyde is included in this series since a possibility exists that auto-oxidation may occur. Addition of oxygen to the aldehyde group takes place, forming perbenzoic acid which oxidizes benzaldehyde to benzoic acid and is itself reduced to the same acid.

Table 8 shows the growth response obtained from certain miscellaneous aromatic compounds.

Table 7. Utilization of benzoic acid, mono hydroxy substituted benzoic acids, and benzaldehyde by the reference cultures and soil isolates.

Organism	Benzoic acid	Ortho-hydroxy benzoic acid	Meta-hydroxy benzoic acid	Para-hydroxy benzoic acid	Benzaldehyde
ATCC 4278	XXXX*	-	-	-	XXXX
ATCC 4279	XXXX	-	-	-	XXXX
41-2	XXXX	-	-	-	XX
53-1A	XXXX	-	-	-	XX
53-1B	XXXX	-	-	-	XX
53-1C	XXXX	-	-	-	XX
53-1D	XXXX	-	-	-	XX
53-2	XXXX	-	-	-	XXXX
91-1	-	-	-	-	-
91-4	XXX	-	-	-	XX
106-1	XX	-	-	-	-
106-2	XX	-	-	-	-
106-5	XXX	-	-	-	XX
106-6	XXX	-	-	-	XX
110-1	XXX	X	-	-	X
110-2	XXX	-	-	-	XXX
110-3	XXX	-	-	-	XXX

* X, slight growth; XX, moderate growth; XXX, good growth; XXXX excellent growth; -, no growth or equal to control.

Table 8. Utilization of certain miscellaneous aromatic compounds by the reference cultures and the soil isolates.

Organism	Aniline	Alpha-naphthol	Diphenyl amine	Naphthalene	Alpha-naphthyl amine
ATCC 4278	XX*	-	-	XX	-
ATCC 4279	XX	-	-	XX	-
41-2	XX	-	-	X	-
53-1A	XX	-	-	XX	-
53-1B	XX	-	-	XX	-
53-1C	XX	-	-	XX	-
53-1D	XX	-	-	XX	-
53-2	XX	-	-	XX	-
91-1	X	-	-	X	-
91-4	XX	-	-	X	-
106-1	XX	-	-	X	-
106-2	XX	-	-	X	-
106-5	X	-	-	XX	-
106-6	XX	-	-	XX	-
110-1	X	-	-	X	X
110-2	X	-	-	XX	-
110-3	X	-	-	XXX	-

* X, slight growth; XX, moderate growth; XXX, good growth; XXXX, excellent growth; -, no growth or equal to control.

Although six membered alicyclic compounds are not aromatic in nature, there is a marked resemblance between the structure of the aromatic benzene nucleus and such alicyclic compounds as cyclohexane.

To determine growth response by Mycoplana species upon representative members of this group, the six carbon compounds cyclohexane and cyclohexene were tested for the ability to serve as a sole carbon source.

Mineral salts agar plates were streaked with washed cell suspensions of the soil isolates and reference cultures. A few milliliters of the compound to be tested were pipetted onto the surface thus flooding the inoculated agar. Bushnell and Haas (1941) used this technique with success in growth experiments using various liquid hydrocarbons and they reported that the procedure did not interfere with growth of their cultures.

No growth was noted with any of the cultures employed thus indicating that the six membered alicyclic cyclohexane and cyclohexene cannot be used as sole carbon sources by these organisms.

Bushnell and Haas (1941) tested the growth response of Mycoplana bullata and M. dimorpha upon mineral salts agar plates using kerosene as the sole carbon source. They reported that no growth was obtained.

DISCUSSION

It has been apparent from the onset of this study that the taxonomic relationship of the genus Mycoplana, with respect to the other genera of the family Pseudomonadaceae, is to be questioned.

It is believed that any taxonomic group should be based upon characteristics which will remain stable and which will not be subject to extreme variation. These characteristics should be readily apparent and well described.

The original description of Mycoplana by Gray and Thornton (1928) was, from a practical viewpoint, wholly inadequate. The methods used to determine biochemical activity were not described in such a manner as to allow for accurate reproducability and were not sufficiently inclusive to give a true picture of the range of biochemical activity. The descriptions concerning the flagellation of the species of Mycoplana are ambiguous and lend definite speculation concerning family status, this fact being recognized by the editors of the Bergey's Manual for Determinative Bacteriology, Sixth Edition.

Several of the characteristics reported for the species of the genus Mycoplana are not entirely commensurate with results obtained in this study. For the most part, these discrepancies have been in the biochemical reactions as were originally described.

It has been shown that both species of Mycoplana are definitely nitrite positive after 24 hours, the test becoming negative after 144 hours. It was stated previously that the test period used by Gray and Thornton was 7 days, yet the presence of a nitrite intermediate can be readily detected for 5 days with the test becoming negative on the sixth. Actually this is in accord with the results obtained by Gray and Thornton, however, the time elapse of the test period used by them is to be criticized.

The genus Mycoplana is separated into its species on the basis of the liquifaction of gelatin. It was originally reported that Mycoplana dimorpha liquified gelatin while M. bullata lacked this ability. This study reveals that both species lack an enzyme system capable of bringing about liquifaction of gelatin. It is to be noted at this point that neither species of Mycoplana possess the ability to hydrolyze starch. It was originally reported that Mycoplana dimorpha was capable of starch hydrolysis, while M. bullata lacked this ability. However, the results obtained in this study lend support to the supposition that the two species are identical, since starch hydrolysis and the lack of gelatin liquifaction are common to both Mycoplana dimorpha and M. bullata.

This study indicates that Mycoplana bullata is able to hydrolyze starch. This is in opposition to the results obtained by Gray and Thornton. This can be explained on the basis of adaptive enzymes; i.e., the stimulatory effect of a given substrate upon the production of the homologous enzyme by the cells in question. A

possibility exists, however, that the gain of this activity was the result of dissociation or variation.

Two conditions arise as possible explanations for the variance obtained in the results obtained in this study and the results obtained by Gray and Thornton. These possibilities are loss of characteristics upon long sub-culturing and the isolations of aberrant strains.

It has been established that microorganisms are subject to variation or dissociation over an extended period of sub-culture. The reference cultures, which were used in this study, were obtained from the American Type Culture Collection, Washington, D. C. They were first deposited in this collection by Dr. H. J. Conn in 1928. It is considered highly possible that the long period of sub-culturing suffered by the reference cultures of Mycoplana could induce characteristics different from those originally described. The original characteristics should be restored, however, when growth conditions well suited for their invigoration are employed. The possibility remains, however, that "invigoration dissociation" may be induced so that the phase subsequently studied may be quite different from the original. The reference cultures were subjected to rapid transfer in nutrient broth using the optimum culture conditions described for these organisms.

Although dissociation of these microorganisms is to be considered, a more logical explanation lies in the possibility that Gray and Thornton described what were possibly aberrant strains.

The Manual of Methods for Pure Culture Study of Bacteria states that the description of a new species should be based on at least six representative isolations of the organism. The genus Mycoplana was originally described as a new genus upon a total of three strains; one strain of Mycoplana dimorpha and two of M. bullata. It is felt that this total is insufficient to warrant adequate evidence of true generic status.

Taxonomically, the placing of Mycoplana in the family Pseudomonadaceae was highly questionable according to the original descriptions by Gray and Thornton. Conclusive evidence has been presented in this thesis to show that these organisms have been correctly placed with regard to the proper family. They satisfy the required characteristics of the family Pseudomonadaceae; i.e., they possess polar flagella and they are Gram negative bacilli. However, it is felt that the genus Mycoplana is invalid since the salient characteristic of branching cells can neither be observed nor reproduced.

It is thus proposed that the species of Mycoplana are in actuality members of the genus Pseudomonas. The following taxonomic relationship is advanced as the possible position occupied by these species:

Abridged Key to the Species of Genus Pseudomonas.

I. Soil and fresh water forms with a few that are pathogenic on cold or warm blooded animals.

1. Green fluorescent pigment not formed.

a. Gelatin not liquified.

b. Polar flagellate.

c. Grow poorly at 37°C.

d. Utilize Hydrocarbons.

e. Pseudomonas bullata.

This tentative relationship is supported by the fact that five species of the genus Pseudomonas originally described by Gray and Thornton occupy a similar position. These species are Pseudomonas desmolyticum, P. rathonis, P. dacunhae, P. arvilla, and P. salopium. (Bergey's Manual of Determinative Bacteriology, Sixth Edition, page 85). The species name M. bullata is retained in preference to M. dimorpha. The term "dimorpha" refers to "two forms" and is not considered as being truly descriptive of the observed morphology of the type cultures. It is felt, however, that a more exhaustive study should be made in regard to the organisms in this group, since the descriptions of the six species are inadequate and incomplete. This deficiency becomes important when correlation is attempted between members of this group and unknown strains having similar characteristics. In too many cases, the lack of adequate information creates doubt concerning correct taxonomic relationships.

The identification of the soil isolates presents several interesting problems. The soil isolates may be subdivided into two general groups. The first group contains those cultures which resemble the reference cultures both biochemically and morphologically. The second group contains those cultures which show distinct differences from the characteristics of the type cultures. It will be remembered that nitrogen production along with a positive nitrite test constituted the prime characteristics for the separation of the soil isolates to be studied. Cultures 106-1 and 106-2 have lost both the ability to reduce nitrates to nitrites and to produce gas in the fermentation vials. Cultures 110-1, 110-2, and 110-3 lost the ability to produce gas but retained the ability to reduce nitrates to nitrites. Upon passage through nitrate broth, cultures 110-2 and 110-3 partially regained the ability to produce gas, while culture 110-1 did not. Cultures 106-1 and 106-2 remained negative in respect to both characteristics when passed through nitrate broth. Cultures 91-1 and 91-4 show several biochemical variations from that of the reference cultures (ATCC 4278 and ATCC 4279), but produce gas in nitrate broth and are nitrite positive.

The loss of the ability to produce gas and reduce nitrates to nitrites of cultures 106-1, 106-2, and 110-1, when grown in nitrate broth, is difficult to explain. It serves, however, to illustrate the ability of microorganisms to vary from the normally expected reaction.

Cultures 110-2 and 110-3 show characteristics quite similar

to those described for Pseudomonas caudata and are tentatively identified as this species. Culture 110-1, although from the same source, exhibits several different properties. It shows a taxonomic relationship to Pseudomonas pictorum; originally described by Gran and Thornton. However, here again the descriptions presented are too inadequate to afford correct taxonomic relationship.

Cultures 91-1 and 91-4 show characteristics which closely parallel those described for Pseudomonas methitica, and they are considered as being closely related to this species.

Cultures 106-1 and 106-2 exhibit characteristics described for Pseudomonas astragali, however, a more complete study would be desirable before assignment of these cultures to P. astragali is attempted.

Cultures 41-2, 53-1A, 53-1B, 53-1C, 53-1D, 53-2, 106-5, and 106-6 show biochemical and morphologic characteristics which are closely parallel to those of the type cultures of the presently accepted species of the genus Mycoplana. They are considered either to be identical with the type cultures (ATCC 4278 and ATCC 4279) or very closely related.

The utilization of certain aromatic compounds by the microorganisms studied in this investigation shows several interesting relationships. Bernheim (1942) reported that the non-pathogenic species of Mycobacterium, used in his study of the oxidation of aromatic compounds, could utilize phenol but not aniline. He states that amino groups will generally inhibit the oxidation

of the benzene ring. The organisms used in this study could metabolize aniline with varying degrees, while diphenylamine and alpha-napthylamine were not attacked. This substantiates, in part, the statement of Bernheim.

The ability of this group of organisms to utilize the hydroxy substituted benzene ring is restricted to phenol, resorcinol, and phloroglucinol, the emphasis of utilization of the polysubstituted ring being on the meta isomers. The ortho and para isomers were not attacked.

No growth was obtained on media containing the isomeric hydroxybenzoic acids as sole sources of carbon. This was unexpected since the hydroxy and carboxy, when separately substituted on the benzene ring, gave excellent growth response. It was presupposed that isomeric preference would be demonstrated, however, this was not the case. The inability of the test organism to metabolize this group of compounds is not known, but it is assumed that it is due to an enzyme system which is lacking.

Isomeric preference is demonstrated in the case of the monohydroxy substituted toluenes. Consistent growth was obtained only on the ortho isomer while sporadic growth response was obtained with the meta and para isomers. It was noted that the meta isomer (m-cresol) was metabolized by only four of the test organisms and that the growth response was slight. However, 3,5 dihydroxytoluene (orcinol) supported good growth and was metabolized by all of the test organisms.

SUMMARY

Fifteen cultures of organisms having the ability to utilize the benzene ring as a sole source of carbon were isolated from five soil samples out of the 124 samples screened.

These organisms were studied with regard to their taxonomic relation to the genus Mycoplana.

The utilization spectrum of the type cultures of Mycoplana bullata, M. dimorpha, and of the soil isolates was extended to several simple benzene derivatives. The substances tested were those simple compounds which contained no more than one carbon atom in the substituted group or groups.

The presently accepted genus Mycoplana has been logically established as a member of the family Pseudomonadaceae. The flagellation of these species was shown to be polar with motrichous forms predominating.

Several biochemical and morphological differences have been pointed out as being in opposition to characteristics originally described for the genus Mycoplana.

It is suggested that the presently accepted genus Mycoplana is invalid since the prime separation characteristic of branching of the cells can neither be observed nor reproduced. It is felt that a close relationship exists between the type species of Mycoplana and the other members of the genus Pseudomonas. An abridged key is presented to show the proposed classification of the species of the now accepted genus Mycoplana in the genus Pseudomonas.

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STUDIES ON THE GENUS MYCOPLANA

by

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ABSTRACT

The presently accepted genus Mycoplana was originally described by Gray and Thornton (1928). They studied a heterogenous group of microorganisms which had the ability to utilize phenol as a sole carbon and energy source. This group of organisms contained, in addition to the genus Mycoplana, members of Micrococcus, Mycobacterium, Bacterium, Pseudomonas, Vibrio, and Bacillus. The genus Mycoplana was described as a new genus by virtue of the branching character of the cells. At that time, Gray and Thornton tentatively placed Mycoplana in the family Mycobacteriaceae because of the characteristic of branching cells. However, the Editors of Bergey's Manual later moved the genus to the family Pseudomonadaceae. This change was necessitated by the fact that the cells of Mycoplana are non acid fast, Gram negative, and are motile by means of polar flagella. The prime morphologic character which taxonomically separates Mycoplana from the other genera of the family Pseudomonadaceae is branching of cells, and secondarily the utilization of phenol.

Fifteen cultures of organisms having the ability to utilize phenol as a sole carbon source were isolated from five soil samples out of 124 screened. These cultures were studied with regard to their taxonomic relationship to the genus Mycoplana.

Studies on the morphologic characteristic of flagellation revealed that the cells were polar flagellated, with monotrichous forms predominating. This type of flagellation and the Gram negative character warranted placement of this genus in the family Pseudomonadaceae.

The prime characteristic of branching cells could not be naturally observed in this research, and since this characteristic separates Mycoplana from certain species of the genus Pseudomonas, considerable doubt existed with respect to the validity of the genus Mycoplana. Attempts to produce branching by changing culture and environmental conditions were unsuccessful. The methods used in an attempt to produce branching were: Variation in pH; altering temperature of incubation; testing effect of reduced surface tension; testing effect of phenol on cell morphology; age of culture; testing effect of reduced oxygen tension; employing varying amounts of NaCl in the medium; studying the effect of crystal violet; studying the effect of osmotic pressure by using varying amounts of glucose, and studying the effect of 10 percent lithium chloride on cell morphology.

The utilization spectrum for carbon sources of type cultures of Mycoplana dimorpha and M. bullata was extended to several simple benzene derivatives. The test organisms were grown upon a mineral salts agar base having no available carbon source other than that of the aromatic compound to be tested. The substances tested were those simple compounds which contained no more than one carbon atom in the substituted group or groups. This minimized the possibility of the carbon atoms of an extended side chain being oxidized in preference to the carbon atoms of the benzene ring. The aromatic compounds tested were phenol, catechol, resorcinol, hydroxyquinone, pyrogallic acid, phloroglucinol, toluene, o-cresol, m-cresol, p-cresol, orcinol, benzoic acid, O-hydroxybenzoic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, benzaldehyde,

aniline, alpha-naphthol, diphenylamine, naphthalene, and alpha-naphthylamine. Growth response was obtained on phenol, resorcinol, phloroglucinol, o-cresol, orcinol, benzoic acid, benzaldehyde, aniline, and naphthalene. No growth was obtained on the other compounds mentioned. The ability of the test organisms to metabolize alicyclic compounds resembling the benzene ring were tested by growth experiments using cyclohexane and cyclohexene as the sole source of carbon. These compounds were not able to be metabolized by the test organisms.

It was noticed that in the case of the hydroxy substituted phenols, only the meta isomers were utilized. If the meta isomer of hydroxy benzoic acid had been utilized, a postulation of a group-position factor could be made. However, no growth occurred on m-hydroxybenzoic acid after 72 hours incubation. Isomeric preference was demonstrated in the case of the mono-hydroxy substituted toluenes. Consistent growth was obtained only on the ortho isomer, however, 3,5 dihydroxytolune (orcinol) supported good growth by the test organisms.

Several biochemical differences were noted which were in opposition to those originally described by Gray and Thornton for the genus Mycoplana. This study indicates that the two species of Mycoplana are biochemically identical.

The results found in this study suggest that the presently accepted genus Mycoplana is invalid, and that the species of the genus Mycoplana are actually members of the genus Pseudomonas. This is suggested since the salient characteristic of branching could neither be observed nor reproduced. The following taxonomic

relationship is advanced as the possible position occupied by the species of Mycoplana to the species of Pseudomonas:

Abridged Key to the Species of Pseudomonas.

I. Soil and fresh water forms with a few that are pathogenic in cold or warm blooded animals.

1. Green fluorescent pigment not produced.

a. Gelatin not liquified.

b. Polar flagellate.

c. Grow poorly at 37°C.

d. Utilize hydrocarbons.

e. Pseudomonas bullata.

This tentative relationship is supported by the fact that five species of the genus Pseudomonas originally described by Gray and Thornton occupy a similar position. These species are Pseudomonas desmolyticum, P. rathonis, P. dacunhae, P. arvilla, and P. salopium. The species name M. bullata is retained in preference to M. dimorpha. The term "dimorpha" refers to "two forms" and is not considered as being truly descriptive of the observed morphology of the type cultures.