A SELECTIVE MEDIUM FOR THE ISOLATION OF PSEUDOMONAS AERUGINOSA

by

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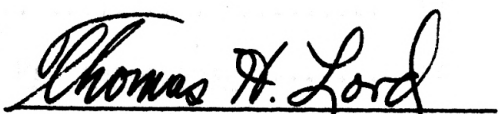

Major Professor

TABLE OF CONTENTS

INTRODUCTION

Members of the genus Pseudomonas are important because they cause spoilage of foods, especially meat and eggs; also some members are pathogenic to plants and animals. They are widespread in soil, fresh and sea water, water from sulfur springs and putrefying matter. Pseudomonas is one of the gram negative organisms frequently isolated in diagnostic laboratories from clinical cases. Although pseudomonads usually are not primary pathogens, they are recognized as frequent secondary invaders.

According to Breed et al. (1957) many Pseudomonas species form a diffusible pigment, but many Pseudomonas species lose the property of forming pigment on continued subculture and some never appear to have possessed it. This thesis concerns the development of a selective medium for the isolation of the Pseudomonas aeruginosa from different sources, e.g., water, sewage, animal and human feces, river and pond water, soil, and food products.

Also the effect of pyocyanin as a bactericidal agent on different types of organisms was studied. Specific antibiotics and quaternary ammonium compounds were chosen which in high concentration had imperceptible effect on Ps. aeruginosa yet inhibited other organisms in low concentrations. This selective mechanism was applied to the study of the incidence of Ps. aeruginosa in natural sources.

REVIEW OF LITERATURE

The genus Pseudomonas was described by Migula (Breed et al., 1957). It is composed of gram negative, monotrichous, lophotrichous or non-motile bacilli. They frequently develop fluorescent, diffusible pigments of a

greenish, bluish, lilac, rose, yellow or other color. Sometimes the pigments are non-diffusible, bright red or yellow. There are many species that fail to develop any pigmentation or lose their ability to produce pigments upon subculturing. The majority oxidize glucose to gluconic acid. Their ability to break down the compound arginine was used as a feature for identification of Pseudomonas strains which do not produce pigments (Sherris et al., 1959). Lactose is not fermented. Nitrates are reduced to nitrites, ammonia or free nitrogen. Some species attack hydrocarbons.

A basal medium for culturing Pseudomonas has been studied by many workers, especially for the essential requirements to produce maximum pyocyanin pigment. Gessard (1890, 1891, 1892) found that media containing glycerol, peptone, and agar produce great amounts of pyocyanin. Jordan (1899) stated that pyocyanin production was not affected by phosphates or sulfates and that ammonium salts of succinic, lactic, acetic or citric acids can be used as the sole source of carbon and nitrogen. In 1943, Seleen and Stark verified that the best medium for pyocyanin formation is the Gessard's glycerol peptone medium.

Burton et al. (1947), using Gessard's glycerol peptone medium as a control, found that alanine or glycine, leucine and also K^+, PO_4^{---}, Fe^{++} and Mg^{++} were essential for pigment production. In their work a basal medium was used which was a modification of the synthetic medium developed by Burton et al. (1947, 1948) and also by Ringen and Drake (1952). This basal medium contained glycerol, L-leucine, K_2HPO_4, $FeSO_4 \cdot 7\ H_2O$, and $MgSO_4 \cdot 7\ H_2O$. Harris (1950) found that pyocyanin was produced late in the culture cycle and accumulated as a waste product of metabolism. Ringen and Drake (1952) added pyocyanin to this basal medium to produce a selective medium for the isolation

of Ps. aeruginosa from other organisms from different sources. This selective medium was then applied to a study of the incidence of Ps. aeruginosa in natural sources.

Action of Pyocyanin

Fredericq (1946) by streak tests on agar plates found that Pseudomonas aeruginosa inhibited the growth of numerous species of bacteria.

Young (1947) found that acid pyocyanin, when added to the medium, has a bactericidal action against other organisms, as Staphylococcus aureus, Mycobacterium smegmatis, but not against Escherichia coli.

Action of Antibiotics

Pseudomonas species are considered among the most resistant to antibiotics and chemotherapeutic drugs. The effect of antibiotics on Pseudomonas and other organisms has been studied since the discovery of antibiotics. The antibiotics which have been studied on Pseudomonas are: Penicillin, dihydrostreptomycin, chloromycetin, erythromycin, aureomycin, terramycin, polymyxin-B, oxytetracycline, neomycin, viomycin, bacitracin, tyrothricin, and xanthocellin, etc. All these antibiotics were used by different workers singly or in combination.

Ellard (1953) stated that susceptibility of Pseudomonas to antibiotics depends upon the medium in which the inoculum was originally grown and the medium in which the organisms were exposed to the antibiotics. Erlanson (1952) found that solid media seemed to be preferable, and diffusion methods were simpler to perform than dilution methods. Streak, dilution and disc methods have been applied for the study of the action of antibiotics. Wright

et al. (1954) found that Pseudomonas species that produce neither fluorescent pigmentation nor pyocyanin were more sensitive to antibiotics. Bekierkunst (1950), working on Pseudomonas resistant to streptomycin, concluded that cells grown on a synthetic medium were greater in sensitivity to antibiotics than ones grown in broth, i.e., resistant strains developed much faster in broth than in a synthetic medium. Weiss and Hochfeldt (1957) stated that cultures grown at 30°C were more resistant than those grown at 37°C.

Wright et al. (1954), working on the susceptibility of Pseudomonas to ten antibiotics in vitro, concluded that all strains were resistant to high concentrations of bacitracin and penicillin (400 µg/ml). Tokuji (1954) found that oxytetracycline had slight antibacterial action on Ps. aeruginosa ranking next to polymyxin-B, and it is more active than tetracycline and chlorotetracycline, while Henneberg and Muller (1954) stated that each strain of the 16 strains of Ps. aeruginosa they used had different degrees of sensitivity to the antibiotic effect of streptomycin, chloromycetin, terramycin, and polymyxin. Robinson et al. (1944), working on the chemotherapeutic properties of streptomycin on gram negative and gram positive bacteria, found that Ps. aeruginosa showed resistance to this antibiotic. Arquie et al. (1948) found that certain strains of Ps. aeruginosa inhibited cultures of Actinomyces griseus and inactivates streptomycin due to the production of streptomycinase. While Gumbach (1951) stated that Ps. pyocyanea filtrate inactivated streptomycin and activated penicillin. Bekierkunst (1951), working on 24 strains of Ps. aeruginosa, found that streptomycin in concentrations of 5 to 125 µg/ml inhibits growth of Pseudomonas. Yow, et al. (1953) worked on the sensitivity of 100 strains of Ps. aeruginosa to penicillin, streptomycin, chlortetracycline, chloramphenicol, oxytetracycline, neomycin, bacitracin and polymyxin-B.

Polymyxin-B inhibits growth of all strains. Terramycin, neomycin and streptomycin were less consistently inhibitory, and the others showed minimum effect. Wright et al. (1954) found that polymyxin-B is the most inhibitory agent and most all the strains of Pseudomonas they tested were inhibited by a concentration of 6.3 µg/ml or less. Frank (1950) stated that aerosporin and polymyxin were effective on Ps. aeruginosa, while Tropnel (1954) found that Pseudomonas infections were successfully treated with polymyxin.

Daikos et al. (1960) found that vancomycin in concentrations from 0.075 to 20 ug/ml did not affect four strains of Ps. aeruginosa. Armstrong and Larner (1951) worked on the effect of combinations of antibiotics on 7 strains of Ps. aeruginosa and on Proteus vulgaris in vitro and in vivo. They found the following bactericidal concentrations:

Penicillin	4,700 µg/ml
Aureomycin	250 µg/ml
Chloramphenicol	25-250 µg/ml
Dihydrostreptomycin	125-250 µg/ml
Terramycin	62-250 µg/ml

Kietzmann (1955) found that polymyxin and terramycin gave the strongest inhibitory action followed by streptomycin.

Effect of Fluoride and Acids

Miyoshi (1955), studying the effect of sodium fluoride on various bacteria, stated that Pseudomonas was sensitive to small doses in relation to other organisms. Angelillo (1955) found that Ps. aeruginosa was very

sensitive to acetic acid, mandelic and boric acid in concentrations of 0.1 to 0.3 µg/ml.

Effect of Quaternary Ammonium Compounds

Hucker et al. (1947, 1948) found that E. coli can be killed completely by the quaternary ammonium compounds they studied while Ps. fluorescens is the most resistant of the vegetative cells studied. They studied the relative germicidal rate against bacterial vegetative cells and spores by using thirteen quaternary ammonium salts: cetavlon, QB, onyxide quartol, isothan OX, Napco Q Cl, Hyamine 1622, Hyamine 10X, B.T.C. Roccal, Tetrosan, ceepryn, emulsept, Isathan Q15, and Isothan Q4. Ps. fluorescens was the most resistant of the vegetative types. They studied the effect of organic matter, e.g., horse serum, skim milk, cottonseed oil and soluble starch, upon the germicidal activity of nine quaternary ammonium salts. The effect of skim milk was greater than the others which shows that the proteins of milk are absorbing more quaternary ammonium cations than horse serum, sugars and other organic constituents of milk. The effect of hydrogen ion concentration on a series of ten representative quaternary ammonium compounds was also investigated by Hucker et al. (1948). Buffered media were used having a range of pH from 2 to 9.0 but clear results did not appear in all cases. They stated that of the ten quaternary ammonium compounds studied, practically all were more active either in the alkaline or in the acid range.

Effect of Sodium Chloride

Pseudomonas species are able to grow on sodium chloride up to three percent, as stated by Shimwell et al. (1960).

Effect of Vibriostatic Agents

Shewan and Hodgkiss (1954) developed a method for the rapid differentiation of certain non-pathogenic asporogenous bacilli by using certain vibriostatic agents to differentiate between Pseudomonadaceae members and other organisms. Pseudomonas was found to be insensitive to vibriostatic agents as 0/129 2,4 diamino 6-7 di-isopropyl pteridine and its 6-7 diethyl derivative. The action of a series of pteridines was studied by Collier et al. (1950) on the inhibition of growth of Streptococcus faecalis, Lactobacillus casei, Lactobacillus arabinosus, and other organisms and showed that 0/129 had the highest activity of all compounds tested.

EXPERIMENTAL METHODS

Preparation of Basal Media

The liquid medium used was a modification of one by Burton et al. (1948) and is composed of the following:

glycerol	1.0 gm
glycine	0.6 "
L-leucine	0.6 "
$K_2H\ PO_4$	0.04 "
$MgSO_4 \cdot 7\ H_2O$	2.0 "
$FeSO_4 \cdot 7\ H_2O$	0.001 "

The above ingredients were mixed in 100 ml distilled water and heated to dissolve. The pH was adjusted to 7.4 to 7.6. Agar agar was added in 3 percent amount to obtain a solid medium. Both media were sterilized at 121^{o}C for 15 minutes.

Pyocyanin Medium

Ten ml of the sterile liquid basal medium was put aseptically into 100 ml sterilized Erlenmeyer flasks, inoculated with a loopful of Ps. aeruginosa broth culture which produced pyocyanin. Incubation was at 30°C with continuous shaking until maximum pyocyanin was formed. This usually took 3 to 4 days. Shaking maintained the aerobic condition and maximum production of pyocyanin. The pyocyanin was extracted with chloroform. It was removed from the chloroform with 0.1N HCl. The intensity of the acid aqueous pyocyanin solution was standardized by using a Bausch and Lomb Spectronic 20 spectrophotometer at wave length 625 mu so that it gave 5% transmission. This pyocyanin solution is equal to 0.45 mgm pyocyanin/ml.

For preparing the pyocyanin medium, the ingredients of the basal medium were dissolved in 80 ml distilled water instead of 100 ml and 20 ml of neutral pyocyanin solution was added. The medium was adjusted to pH 7.4 to 7.6.

The solid and liquid basal media and also the pyocyanin medium were tested for the growth of five species of Pseudomonas, by using the streak technique on plates of the solid medium. The five species used were: Ps. cruciviae, Ps. aeruginosa strain A, Ps. sp., Ps. indoloxidans, and Ps. aeruginosa strain B.

Incubation was at 30°C for 48 hours. Nutrient agar and nutrient broth were used as controls for the type of growth of these Pseudomonas species. Gram stains were used for the study of their morphology.

Study of the Action of Antibiotics and Quaternary Ammonium Compounds

To study sensitivity of five species and strains of Pseudomonas to these compounds, basal agar medium plates were prepared, each containing different concentrations of the antibiotics and quaternary ammonium compounds singly and in combinations.

A heavy inoculum of organisms was streaked on each plate and incubated at 30°C for 48 hours, and growth or inhibition were recorded.

The penicillin used in this work was Penicillin GK Squibb buffered with sodium citrate, in bottles of 500,000 units, diluted with sterile distilled water to fulfill the required dilutions.

Streptomycin Sulfate, Squibb, was used, packaged with each bottle containing 5 gm pure streptomycin base. The quaternary ammonium compounds used during this study were:

1. B.T.C. U.S.P. 50* with the following composition:

n-alkyl (50% C_{12})	
(3% C_{14})	
(17% C_{16})	
(3% C_{18})	
Benzyl ammonium chloride	50%
Inert ingredients	50%
	100.00

2. Quadrol: N_1N_1N', N' - tetraKis (2-hydroxypropyl) ethylene diamene.**

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* Produced by Onyx Chemical Corporation, Jersey City, New Jersey.
** Produced by Wyandotte Chemical Corporation, Wyandotte, Michigan.

3. Hyamine 10X***: the active ingredient is Diisobutyl cresoxyethoxy-ethyl benzyl ammonium chloride monohydrate.

4. Hyamine 1622***: the active ingredient is Paradiisobutyl phenoxymonohydrate.

Different dilutions of the above quaternary ammonium compounds were prepared in sterilized distilled water and flasks. After a week samples from these diluted quaternary ammonium compounds were inoculated in duplicate onto nutrient agar and into nutrient broth. Incubation was at 30°C and 37°C for 48 hours. Growth of organisms was checked to be certain that the diluted quaternary ammonium compound solutions were self sterilized.

A study was made of the action of certain concentrations of four quaternary ammonium compounds singly and in combination with antibiotics on the growth of the bacteria. The following four concentrations of quaternary ammonium salts were added singly or in combination to a basal agar medium containing 400 mg/ml penicillin and 6 mg/ml streptomycin:

BTC	= 1:10,000
Quadrol	= 1:1,250
Hyamine 10X	= 1:3,000
Hyamine 1622	= 1:3,000

Streaking and inoculation were done as above and incubation was at 30°C and 37°C for 48 hours.

*** Produced by Rohm and Haas, Philadelphia, Pa.

Study of the Action of Basal Agar Medium Containing Different Concentrations of Antibiotics and Quaternary Ammonium Compounds on Ps. aeruginosa and Other Bacteria

The basal agar medium was used as a base. Pyocyanin, different concentrations of antibiotics and quaternary ammonium salts were added singly or in combination to plates of this basal agar medium. The sensitivity of Ps. aeruginosa and other microorganisms was tested by streaking a heavy inoculation of these bacterial cultures on duplicate plates containing these constituents. Incubation was at 30°C and 37°C for 48 hours. Growth and inhibition were noted.

Isolation of Ps. aeruginosa from Different Natural Sources by Using the Above Selective Medium

Samples of natural materials, such as untreated and treated sewage, mastitis milk, river water, fish pond water, human and animal feces, and soil were used in this study.

The basal agar and liquid medium were used containing the following:

Penicillin	100 µg/m
Streptomycin	1.5 µg/ml
BTC	1:40,000
Quadrol	1:5,000
Hyamine 10X	1:12,000
Hyamine 1622	1:12,000

One ml of each material from natural sources was added to 10 ml of the above liquid medium in 100 ml Erlenmeyer flasks. Incubation was for 48 hours at 30°C with continuous shaking as a process of enrichment. After enrichment

0.1 ml of each culture was streaked by a loop over plates containing the above medium with 3% agar, and incubated at 30°C for 48 hours. Growth of organisms and pigment production were recorded. For a control a loop of broth culture of Ps. aeruginosa was inoculated into the above liquid medium already containing 1.0 ml of material from natural sources, incubated with shaking at 30°C for 48 hours. The enrichment culture was subcultured onto basal agar medium plates and growth and pigment production were observed after incubation at 30°C for 48 hours. Similarly the materials from natural sources were inoculated into nutrient broth, incubated at 30°C for 24 hours, then subcultured onto Gessard's glycerol peptone agar as a control. Also the samples from the enrichment selective basal broth were subcultured onto Gessard's glycerol peptone agar containing the same amounts of antibiotics and quaternary ammonium salts as in the basal medium, as a comparison for Ps. aeruginosa growth and pigment production.

EXPERIMENTAL RESULTS

Pyocyanin Action

Table 1 shows that pyocyanin was highly selective for Pseudomonas aeruginosa and inhibited growth of other organisms. It also shows that good growth and pigment production appeared for the Ps. aeruginosa on basal agar or broth media. Nutrient agar and nutrient broth were used as control media for growth and pyocyanin production. The basal medium was better than nutrient agar for optimum growth and pyocyanin production.

Table 1. Amount of growth and pyocyanin production of Ps. aeruginosa as influenced by choice of medium.

Media	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
Liquid Basal Medium					
growth	+++	+++	+++	+++	+++
pyocyanin production	-	+++	-	-	+++
Agar Basal Medium					
growth	+++	+++	+++	+++	+++
pyocyanin production	-	+++	-	-	+++
Pyocyanin Agar Basal Medium					
growth	+++	+++	+++	+++	+++
pyocyanin production	-	?	-	-	?
Nutrient Agar					
growth	+++	+++	+++	+++	+++
pyocyanin production	-	+	-	-	+
Nutrient Broth					
growth	+++	+++	+++	+++	+++
pyocyanin production	-	+	-	-	+

\+ Fair amount of growth or pyocyanin production.
++ Medium amount of growth or pyocyanin production.
+++ Maximum amount of growth or pyocyanin production.
? Indeterminate because medium contains pyocyanin as an ingredient.

Action of Penicillin on Ps. aeruginosa

Table 2 shows that all Pseudomonas strains studied grew well up to 400 µg/ml of penicillin in the medium.

Action of Streptomycin Concentrations on Ps. aeruginosa

As shown in Table 3, all Pseudomonas strains grew well up to 6 µg/ml of streptomycin.

The Action of the Combination of Penicillin and Streptomycin on Ps. aeruginosa

The results of this study are shown in Table 4, where 400 µg/ml penicillin and 6 µg/ml streptomycin could be used in the basal agar medium without inhibitory effects to most Pseudomonas species. Also Ps. aeruginosa appeared to be the most resistant organisms.

Effect of Quaternary Ammonium Compounds on Ps. aeruginosa

As shown in Table 5, all the Pseudomonas species studied could grow in the basal agar medium containing BTC in dilutions greater than 1:20,000. Only Ps. aeruginosa could grow in concentrations as great as 1:250.

Table 6 shows that the Pseudomonas sp. was inhibited in concentrations greater than 1:3,000 in Quadrol, while Ps. aeruginosa could grow in concentrations as great as 1:25.

Table 7 shows that Hyamine 10X in concentrations of 1:2,000 or less allowed all Pseudomonas species to grow. It did not inhibit Ps. aeruginosa even in a concentration of 1:10.

Table 2. Resistance of Pseudomonas strains to penicillin in basal agar medium.

Penicillin Concentration µg/ml media	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
100	+++	+++	+++	+++	+++
200	+++	+++	++	+++	+++
300	+++	+++	++	+++	+++
400	+++	+++	++	+++	+++
500	-	+++	++	+++	+++
600	-	+++	-	+++	+++
800	-	+++	-	+++	+++
1,000	-	+++	-	+++	+++
1,200	-	+++	-	+++	+++
1,500	-	+++	-	+++	+++
2,000	-	+++	-	+++	+++
2,500	-	+++	-	++	+++
3,000	-	+++	-	-	+++
3,500	-	+++	-	-	+++
4,000	-	+++	-	-	+++
5,000	-	+++	-	-	+++
10,000	-	+++	-	-	+++

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 3. Resistance of Pseudomonas species to streptomycin.

Streptomycin Concentration µg/ml media	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
1.0	+++	+++	+++	+++	+++
1.5	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++
4	+++	+++	+++	+++	+++
5	+++	+++	+++	+++	+++
6	+++	+++	+++	+++	+++
10	-	+++	-	+++	+++
15	-	+++	-	+++	+++
20	-	++	-	+++	-
30	-	++	-	+++	-
40	-	++	-	+++	-
60	-	++	-	+++	-
80	-	-	-	-	-
100	-	-	-	-	-
150	-	-	-	-	-
200	-	-	-	-	-
250	-	-	-	-	-
300	-	-	-	-	-

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 4. Combinations of penicillin and streptomycin and their bactericidal action on Pseudomonas species.

Penicillin µg/ml (P) and Streptomycin µg/ml (S)	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
50 P and 0.5 S	+++	+++	+++	+++	+++
100 P " 1.0 S	+++	+++	++	+++	+++
150 P " 1.5 S	+++	+++	++	+++	+++
200 P " 2.0 S	+++	+++	+	+++	+++
250 P " 2.5 S	+++	+++	+	+++	+++
300 P " 3.0 S	+++	+++	-	+++	+++
350 P " 3.5 S	++	+++	-	+++	+++
400 P " 4.0 S	++	+++	-	+++	+++
500 P " 5.0 S	++	+++	-	+++	+++
600 P " 6.0 S	++	+++	-	+++	+++
700 P " 7.0 S	-	+++	-	+++	+++
800 P " 8.0 S	-	+++	-	-	+++
900 P " 9.0 S	-	+++	-	-	+++
1000 P " 10.0 S	-	+++	-	-	+++
1500 P " 15.0 S	-	+++	-	-	+++
2000 P " 20.0 S	-	+++	-	-	+++
2500 P " 25.0 S	-	+++	-	-	+++
3000 P " 30.0 S	-	+++	-	-	+++
3500 P " 35.0 S	-	+++	-	-	+++
4000 P " 40.0 S	-	+++	-	-	+++
4500 P " 45.0 S	-	+++	-	-	+++
5000 P " 50.0 S	-	++	-	-	+++

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 5. Action of different dilutions of quaternary ammonium compound, BTC U.S.P. 50, on the Pseudomonas species.

BTC Dilution	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
1:20,000	+++	+++	+++	+++	+++
1:10,000	+++	+++	+++	-	+++
1:8,000	+++	+++	++	-	+++
1:6,000	+++	+++	++	-	+++
1:5,000	+++	+++	-	-	+++
1:4,000	-	+++	-	-	+++
1:3,000	-	+++	-	-	+++
1:2,000	-	+++	-	-	+++
1:1,000	-	+++	-	-	+++
1:800	-	+++	-	-	+++
1:500	-	+++	-	-	+++
1:250	-	+++	-	-	+++
1:200	-	-	-	-	-
1:150	-	-	-	-	-
1:100	-	-	-	-	-
1:50	-	-	-	-	-
1:25	-	-	-	-	-

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 6. Action of different dilutions of quaternary ammonium compound, Quadrol, on growth of Pseudomonas species.

Quadrol Dilution	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
1:10,000	+++	+++	+++	+++	+++
1:8,000	+++	+++	+++	+++	+++
1:6,000	+++	+++	+++	+++	+++
1:5,000	+++	+++	+++	+++	+++
1:4,000	+++	+++	+++	+++	+++
1:3,000	+++	+++	+++	+++	+++
1:2,000	+++	+++	-	+++	+++
1:1,500	+++	+++	-	+++	+++
1:1,000	+++	+++	-	+++	+++
1:500	+++	+++	-	+++	+++
1:250	+++	+++	-	+++	+++
1:200	+++	+++	-	+++	+++
1:150	+++	+++	-	-	+++
1:100	+++	+++	-	-	+++
1:50	+++	+++	-	-	++
1:25	-	++	-	-	++
1:10	-	-	-	-	-
1:5	-	-	-	-	-

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 7. Action of different dilutions of quaternary ammonium compound, Hyamine 10X, on growth of Pseudomonas species.

Hyamine 10X Dilution	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
1:4,000	+++	+++	+++	+++	+++
1:3,000	+++	+++	+++	++	+++
1:2,000	+++	+++	+++	++	+++
1:1,500	++	+++	+++	-	+++
1:1,000	-	+++	+++	-	+++
1:750	-	+++	-	-	+++
1:500	-	+++	-	-	+++
1:250	-	+++	-	-	+++
1:200	-	+++	-	-	+++
1:100	-	+++	-	-	+++
1:50	-	+++	-	-	+++
1:25	-	+++	-	-	+++
1:10	-	+++	-	-	+++

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 8 shows that the Pseudomonas species could grow in concentrations of 1:2,000 or less of Hyamine 1622. Ps. aeruginosa tolerated concentrations as great as 1:5.

The Action of Four Quaternary Ammonium Compounds Singly or in Combination on Growth of Bacteria

The results in Table 9 show that the microorganisms other than Pseudomonas were more sensitive to the action of a combination of the four quaternary ammonium compounds than Pseudomonas species. Ps. aeruginosa was least sensitive.

Effects of Antibiotics, and Quaternary Compounds on Ps. aeruginosa and Other Microorganisms

The results in Table 10 show that Medium 4 and Medium 6 could be used as selective media for Pseudomonas species, especially Ps. aeruginosa which was the least sensitive organism. Pseudomonas species which produce pigments were more resistant than those which did not produce pigments.

Isolation of Ps. aeruginosa from Different Natural Sources by Using the Above Selective Medium

Table 11 shows the number of samples from different sources and the number of instances Ps. aeruginosa were isolated. The Ps. aeruginosa isolated produced blue-green or lilac pigments in both enrichment broth media and selective agar plates. The plates also had the odor of trimethylamine.

Table 8. Action of different dilutions of quaternary ammonium compound, Hyamine 1622, on growth of Pseudomonas species.

Hyamine 1622 Dilution	Ps. cruciviae	Ps. aeruginosa (strain A)	Ps. sp.	Ps. indol-oxidans	Ps. aeruginosa (strain B)
1:4,000	+++	+++	+++	+++	+++
1:3,000	+++	+++	+++	++	+++
1:2,000	++	+++	+++	++	+++
1:1,500	++	+++	+++	-	+++
1:1,000	-	+++	+++	-	+++
1:750	-	+++	-	-	+++
1:500	-	+++	-	-	+++
1:250	-	+++	-	-	+++
1:200	-	+++	-	-	+++
1:100	-	++	-	-	+++
1:50	-	++	-	-	+++
1:25	-	++	-	-	++
1:10	-	++	-	-	+
1:5	-	++	-	-	+

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 9. The action of quaternary ammonium compounds singly or in combination in basal medium containing penicillin 400 µg/ml and streptomycin 6 µg/ml on different microorganisms.

Constituents added to basal agar medium + penicillin 400 µg/ml + streptomycin 6 µg/ml	Pseudomonas aeruginosa (strain A)	Escherichia coli	Serratia marcescens	Proteus rettgeri	Alcaligenes faecalis	Sarcina flava	Aerobacter cloacae	Staphylococcus aureus	Flavobacterium suaveolens	Bacillus subtilis	Pseudomonas aeruginosa (strain B)	Bacillus megaterium
BTC (1:10,000)	++	+	+++	-	-	-	-	-	-	-	+++	-
BTC + Hy. 10X	+++	-	+++	-	-	-	-	-	-	±	+++	-
BTC + Hy. 1622	++	-	+++	-	-	-	-	-	-	-	++	-
BTC + Quadrol	++	-	+++	-	-	-	-	-	-	-	++	-
Hy. 10X (1:3,000)	++	-	+++	-	-	-	-	-	-	-	++	-
Hy. 10X + Hy. 1622	++	-	+++	-	-	-	-	-	-	-	++	-
Hy. 10X + Quadrol	-	-	+++	-	-	-	-	-	-	-	++	-
Hy. 1622 (1:3,000)	++	-	+++	-	-	+	-	-	-	-	++	-
Hy. 1622 + Quadrol	++	-	+++	-	-	-	-	-	-	-	++	-
Quadrol (1:1,250)	+++	-	+++	-	-	-	-	-	-	-	++	-
BTC + Hy. 10X + Hy. 1622	++	-	-	-	-	-	-	-	-	-	++	-
BTC + Hy. 10X + Quadrol	++	-	-	-	-	+	-	-	-	-	++	-
BTC + Hy. 1622 + Quadrol	-	-	-	-	-	-	-	-	-	-	++	+
Hy. 1622 + Hy. 10X + Quadrol	++	-	-	-	-	-	-	-	-	-	++	-
Hy. 1622 + BTC + Quadrol	++	-	+++	-	-	-	-	-	-	-	++	++
Hy. 1622 + Hy. 10X + BTC + Quadrol	++	=	-	-	-	-	-	-	-	-	++	-

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 10. The growth of microorganisms and Pseudomonas species on basal medium containing different concentrations of antibiotics and quaternary am monium compounds.

Constituents added to basal medium	Pseudomonas cruciviae	Pseudomonas aeruginosa (strain A)	Pseudomonas sp.	Pseudomonas indoloxidans	Pseudomonas aeruginosa (strain B)	Escherichia coli	Serratia marcescens	Proteus rettgeri	Alkaligenes faecalis	Sarcina flava	Aerobacter cloacae	Staphylococcus aureus	Bacillus megaterium	Flavobacterium suaveolens	Bacillus subtilis
Medium 1: pyocyanin only	+++	+++	+++	+++	+++	-	++	++	-	++	++	++	++	-	-
Medium 2: Penicillin 400 µg/ml Streptomycin 6 µg/ml BTC 1:10,000 Quadrol 1:1,250 Hyamine 10X 1:3,000 Hy. 1622 1:3,000 Pyocyanin	-	+++	-	+++	+++	-	-	-	-	-	-	-	-	-	-
Medium 3: Penicillin 200 µg/ml Streptomycin 3 µg/ml BTC 1:20,000 Quadrol 1:3,000 Hyamine 10X 1:3,000 Hy. 1622 1:3,000 Pyocyanin	-	+++	-	-	+++	-	-	-	-	++	-	-	-	-	-
Medium 4: Penicillin 400 µg/ml Streptomycin 6 µg/ml BTC 1:10,000 Quadrol 1:1,250 Hyamine 10X 1:3,000 Hy. 1622 1:3,000	-	+++	-	-	+++	-	-	-	-	++	-	-	-	-	-
Medium 5: Penicillin 200 µg/ml Streptomycin 3 µg/ml BTC 1:20,000 Quadrol 1:2,500 Hyamine 10X 1:5,000 Hy. 1622 1:6,000	-	+++	-	-	+++	-	-	-	-	++	-	-	-	-	-

Table 10. (concl.)

Constituents added to basal medium	Pseudomonas cruciviae	Pseudomonas aeruginosa (strain A	Pseudomonas sp.	Pseudomonas indoloxidans	Pseudomonas aeruginosa (strain B)	Escherichia coli	Serratia marcescens	Proteus rettgeri	Alkaligenes faecalis	Sarcina flava	Aerobacter cloacae	Staphylococcus aureus	Bacillus megaterium	Flavobacterium suaveolens	Bacillus subtilis
Medium 6: Penicillin 100 µg/ml Streptomycin 1.5 µg/ml BTC 1:40,000 Quadrol 1:5,000 Hyamine 10X 1:10,000 Hy. 1622 1:10,000	-	++	++	+++	+++	-	++	-	-	++	-	-	-	-	-
Medium 7: Penicillin 50 µg/ml Streptomycin 0.75 µg/ml BTC 1:80,000 Quadrol 1:10,000 Hyamine 10X 1:20,000 Hy. 1622 1:20,000	-	+++	+++	++	+++	-	++	-	-	++	-	++	++	-	-

\+ Fair amount of growth.
++ Medium amount of growth.
+++ Maximum amount of growth.

Table 11. Isolation of Ps. aeruginosa from natural sources.

Sources	Number of samples	Number of samples containing Ps. aeruginosa		
		Basal agar selective medium	G. agar M. plus antibiotics and quaternary ammonium salts	G. agar M. only
Animal feces	16	1	1	0
Human feces	6	0	0	0
Fish pond water	3	0	0	0
Foods	14	0	0	0
Garbage	10	2	2	0
Mastitis milk	18	5	5	1
Surface waters	10	0	0	0
Sewage	45	25	25	3
Soil	24	5	5	0

DISCUSSION

A selective medium for the isolation of Ps. aeruginosa was developed as a modification of the medium of Burton et al. (1948). Haynes (1951) showed that Gessard's medium produced maximum pyocyanin production in most cases as compared to Gessard's stabs. Occasionally pigments occurred in Gessard's medium in few instances where it did not occur in Burton's medium. This study showed that pyocyanin added to the basal medium completely inhibited different microorganisms and corroborated the results of Young (1947) and Burton et al. (1948). The author chose penicillin and streptomycin as

antibiotics to add to the basal medium because of their low activity against Pseudomonas species in relation to other antibiotics. These results agreed with Robinson (1944), Armstrong and Larner (1951), Bekierkunst (1950) and other workers.

Quaternary ammonium compounds were used by Hucker et al. (1948) as selective reagents. They studied the effect of each compound on different mesophilic and thermophilic bacteria and spores. They stated that Ps. fluorescens was the most resistant to each compound. The findings reported here coincide with their work. The use of a combination of quaternary ammonium compounds with penicillin and streptomycin was first studied in this work. Because of the complications of preparing it, pyocyanin saved the time needed for preparing cultures from which pyocyanin would be extracted and purified.

During the course of experimentation, it was found that good results were obtained by applying an enrichment technique with a liquid selective medium and continuous shaking for 24 hours before culturing on plates of a solid selective medium. Direct streaking of the enriched samples on the solid selective medium gave good isolation of Pseudomonas aeruginosa.

Gessard's glycerol peptone agar medium was used as a control, with and without addition of antibiotics and quaternary ammonium compounds. Enriched Gessard's medium isolated Ps. aeruginosa was equally effective as the selective basal medium, but the latter gave brilliant and deeper colored pigments. Gessard's medium alone cannot separate Ps. aeruginosa from natural sources as shown from Table 11. The samples must be enriched first in the selective basal broth medium containing antibiotics and quaternary ammonium salts, with continuous shaking for 24 hours at 30°C, then subcultured on the

devised medium and Gessard's agar medium containing the same amounts of antibiotics and quaternary ammonium salts, incubated for 48 hours at 30°C followed by a further incubation period for another 48 hours at room temperature. Pigments became more brilliant and deeper in color. During the routine work the above technique was used and Gessard's agar medium was used only as a control. This devised medium showed more brilliant and deeper colored pigment production.

In general, the incidence of Ps. aeruginosa from various natural sources collected agreed with the work of Ringen et al. (1952). Sewage can be considered as a normal habitat for Ps. aeruginosa since nearly 60 percent of sewage samples, raw, partially and wholly treated, studied contained Ps. aeruginosa. Ringen (1952) found that Ps. aeruginosa was isolated from only 3 percent of soil samples and never from natural waters. He stated that the natural habitat of Ps. aeruginosa is feces and sewage. It also seemed that the presence of Ps. aeruginosa in horse feces was a matter of chance. The results of this research agree with the results obtained by Ringen. Also this present study has shown that Ps. aeruginosa can be isolated on the medium devised and cultures show production of pyocyanin in optimum amounts.

SUMMARY

1. A selective medium for the isolation of Ps. aeruginosa is described.

2. Pyocyanin was excluded as an ingredient from the selective medium after studying its effect on microorganisms singly and in combination with penicillin, streptomycin and quaternary ammonium compounds.

3. Penicillin and streptomycin were chosen as the best antibiotics to add to the selective medium after studies of their effect on Pseudomonas species and other bacteria. Penicillin and streptomycin were used because the Pseudomonas species could be considered the most resistant organisms to these antibiotics.

4. The action of different quaternary ammonium compounds, singly or in combination, was studied against Pseudomonas species and other bacteria. BTC U.S.P. 50, Quadrol, Hyamine 10X, and Hyamine 1622 were used for this study.

5. Favorable results were obtained by the use of a combination of antibiotics and quaternary ammonium compounds added to the basal medium.

6. This selective medium was studied for the primary isolation of Ps. aeruginosa from natural sources. It was found that these organisms can be isolated from sewage more consistently than from any other source, such as river and pond waters, milk, foods, and soil, and cultures show production of pyocyanin in optimum amounts.

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A SELECTIVE MEDIUM FOR THE ISOLATION OF PSEUDOMONAS AERUGINOSA

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The genus Pseudomonas lives for the most part in soil, water, sewage, feces, foods, etc. A selective medium for isolation of members of this genus from natural sources would be helpful in solving many problems, due to the importance of the genus as pathogens for man and domestic animals.

Pyocyanin has been described as a highly selective agent almost completely inhibiting the competitive growth of other organisms. It was assayed as an ingredient to add to a basal medium for isolation of Ps. aeruginosa from natural sources. Pseudomonas species can be considered among the most resistant microorganisms to antibiotics except polymyxin-B, which is the most active against Pseudomonads. During this work the action of different concentrations of penicillin and streptomycin added to basal agar medium on duplicate plates, singly and in combination, were studied on Pseudomonas species and other bacteria. The sensitivity was tested by streaking heavy suspensions of these organisms on duplicate plates containing the above constituents. Incubation was at 30°C for 48 hours and growth or inhibition was recorded.

In this work the action of each quaternary compound (BTC U.S.P. 50; Quadrol; Hyamine 10X and Hyamine 1622), singly and in different concentrations and also in combination with each other and with penicillin and streptomycin, was recorded for the effect on Pseudomonas species and bacteria. The streak method was used on duplicate plates containing the above constituents added to the basal agar medium.

Pyocyanin was excluded from this selective medium after its action in combination with quaternary ammonium compounds and antibiotics revealed that a satisfactory selective medium could be obtained in its absence. The final formula developed for the selective medium was:

I. Basal medium:

Glycerol	1.00 gm
Glycine	0.60 "
L-leucine	0.60 "
$K_2H\ PO_4$	0.04 "
$MgSO_4 \cdot 7\ H_2O$	2.00 "
$FeSO_4 \cdot 7\ H_2O$	0.001"

The above ingredients were dissolved in 100 ml distilled water and heated to dissolve. The pH was adjusted to 7.4 to 7.6. Agar agar was added in 3 percent amount to obtain a basal solid medium. The medium was autoclaved at 121°C for 15 minutes.

II. To the above basal medium, liquefied and cooled to 45°C, antibiotics and quaternary ammonium compounds were added aseptically to give the following concentrations:

Penicillin	100 µg/ml of basal medium
Streptomycin	1.5 µg/ml " " "
BTC U.S.P. 50	1:40,000 dilution
Quadrol	1:5,000 "
Hyamine 10X	1:12,000 "
Hyamine 1622	1:12,000 "

The selective medium was applied to the isolation of Ps. aeruginosa from natural sources, such as soil, river water, sewage, milk, foods, water from a fish pond, etc. One ml of each of the natural materials was added to the above selective liquid medium (without agar) in 100 ml Erlenmeyer flasks. Incubation was for 48 hours at 30°C with continuous shaking as a process of enrichment. A 0.1 ml of each culture was streaked by a loop over duplicate

plates containing the above selective medium with 3% agar, and incubated at 30°C for 48 hours. Similarly the materials from natural sources were inoculated into nutrient broth, incubated at 30°C for 24 hours, then subcultured onto Gessard's glycerol peptone agar as a control. Also the samples from the enrichment selective basal broth were subcultured onto Gessard's medium containing the same amounts of antibiotics and quaternary ammonium salts as in the basal medium, as a comparison for Ps. aeruginosa growth and pigment production. The growth of organisms and pigment production were recorded. It was found that Ps. aeruginosa can be isolated from sewage regularly which agrees with the survey done by Ringen et al. (1952) and others. Also this present study has shown that Ps. aeruginosa can be isolated on the medium devised and cultures show production of pyocyanin in optimum amounts.