ISOLATION OF PSYCHROPHILIC HALOPHILES FROM THE ANTARCTIC POLAR DESERT

by

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INTRODUCTION

Eight hundred miles from the south pole on the edge of the Ross sea there lies a series of valleys left free of ice by the recession of the polar ice cap. This region is generally located around $77^{o}34'S$ $161^{o}18'E$ and is comprised of a parallel series of valleys lying for the most part in an east-west direction perpendicular to the coast of the Ross Sea. The area is protected from encroachments of the continental ice sheet by a protective chain of dolerite silled mountains running along the edge of the polar ice cap. There is no measurable precipitation on the floor of the valleys, and most of the snow which does accumulate sublimates at the onset of the austral summer. This region can be classified as a desert, and comparatively it is one of the most arid deserts in the world. Such water as is found in the valleys comes from an annual melt of the alpine and piedmont glaciers which provides only a small amount of water to a very limited area for an extraordinarily short polar summer.

The objective of this study was to investigate the microbial population of the soils of this desert area, specifically those bacteria requiring or tolerating conditions of low temperature and high salt concentration. Knowledge about these microorganisms will add new perspectives to biology: current information indicates Mars

has a cold desert environment, and information about halophilic microorganisms may aid in understanding extraterrestrial life forms in soils which are marginally favorable for life. Different habitats in the seas and oceans are under the influence of marked variations in salinity and temperature. Therefore, some insight into microbial systems of cold desert soils may be applicable to studies of benthic sediments. The food industry will continue to be concerned with these two factors in view of food preservation. It is hoped that some of the concepts developed in this work will resolve or at least indicate a direction for resolution of the physiological questions of psychrophilism and halophilism as they apply to diverse microbial systems.

REVIEW OF THE LITERATURE

Psychrophilism was reviewed by Ingraham in 1962 (17) and Farrell and Rose (10) in 1967. Flannery (11) has reviewed some of the recent papers on halophilic bacteria. Reference will be made only to those papers which are directly pertinent to this thesis.

The term 'psychrophile' has been defined by Ingraham and Stokes (19) as a microorganism which is able to produce visible growth within two weeks at or near zero degrees centigrade. This definition places the emphasis upon the minimum growth temperature instead of the optimum or maximum growth temperature. The definition of an obligate psychrophile tendered by Ingraham and Stokes is a microorganism which is able to produce visible growth within two weeks at 0 C and has a maximum temperature of growth not exceeding 20 C. A facultative psychrophile is a microorganism which is able to grow at 0 C within two weeks and also at temperatures greater than 20 C. These definitions permit the segregation of those microorganisms that have a strict growth requirement for cold conditions from those microorganisms able to tolerate cold temperatures but having an optimal growth temperature in the mesophilic range. It is well known that the maximum growth temperature can be a function of the composition of the growth medium. Borek and Waelsch (4) demonstrated that synthesis of phenylalanine,

tryosine, and aspartic acid in Lactobacillus arabinosis may cease before the cell's catabolic systems are affected. Others have demonstrated a similar variance in phenotypic response with change in temperature (7).

There is some evidence to support the hypothesis that psychrophilic microorganisms have heat labile enzymes which are inactivated at mesophilic temperatures. Morita and Burton (26) demonstrated a temperature sensitive malic dehydrogenase in a marine psychrophile. Langridge and Morita (21) proposed isozymatic forms of heat labile malic dehydrogenase from Vibrio marinus, an obligate psychrophile. A temperature sensitive hydrogenase in a psychrophile was found by Updhyay and Stokes (34). Not only was the enzyme more heat labile than its equivalent in a mesophilic organism, but its synthesis did not occur above 20 C which was the maximum growth temperature for the psychrophile. Temperature sensitive formic hydrogenylase was found by Updhyay and Stokes (33), and Purohit and Stokes demonstrated several heat labile enzymes in a psychrophilic species (28). It should be emphasized that the picture of psychrophilism as caused by temperature lability is by no means clear. In some cases there has been no significant difference in heat stability of equivalent enzymes from psychrophiles and mesophiles (18). Ingraham and Baily also observed variation in heat stability with some differences in crude cell preparations

but not with the purified enzymes of psychrophiles and mesophiles. These differences may be reflections of the choice of strict versus facultative psychrophilic bacteria as model systems. More information is needed about the heat lability of protein in different types of microorganisms to settle the point.

Other explanations have been advanced to explain psychrophilism. Hagen et al (14) demonstrated that the temperature sensitive synthesis of the important cell lipids, phosphatidyl ethanolamine and diphosphotidyl glycerol, could not occur above 25 C in some psychrophilic bacteria. The possibility of temperature dependent repression of enzyme synthesis was demonstrated by Updhyay and Stokes (34) in the synthesis of hydrogenase. Hagen and Rose (13) showed that the growth of a psychrophilic Cryptococcus could be obtained for short periods above its maximum growth temperature (30 C) if the organism was preincubated at 16 C. As has been the case with mesophiles and thermophiles, the cell walls of psychrophiles have been found to break down at temperatures above the maxima for growth. Haight and Morita (15) showed that leakage of nonspecific protein, DNA, RNA, and amino acids takes place from a strict psychrophilic marine bacterium at temperatures above 20 C. The psychrophilic phenomenon is variable, and as yet no physiological model has been found to explain its expression in different microorganisms. A strict psychrophile may require

a series of catalytic and structural proteins which are structurally stable for a relatively limited temperature range. However, the problem of constructing a model becomes more involved when wide ranges of growth temperature are possible. A series of isoenzymes with different temperature optima might explain the biochemical activities at diverse temperatures.

Flannery (11) has defined halophilic and nonhalophilic microorganisms in terms of their respective salt requirements or tolerances. Facultative halophiles are those able to grow in salt concentrations of less than 2 per cent, but they grow optimally at concentrations greater than this. Obligate halophiles grow only in concentrations of salt greater than 2 per cent.

In the past it has been the consensus of several investigators that halophiles were dependent upon osmotic pressures to maintain structural integrity (1, 22). Current evidence indicates however, that some cells have minimum requirements for ions such as sodium, and that high osmotic pressures play a less important role. Recent investigations have emphasized the differing effects of cations on cells, both structurally and biochemically. Abram and Gibbons (2) have postulated electrostatic mechanisms in cell wall integrity of halophiles as has Marquis for nonhalophiles (24). Further, Kates et al (20) have shown sodium dependent synthesis of cell wall precursers. Sodium requirements for other enzymatic activities

have been investigated by Baxter and Gibbons (3) who demonstrated several halophilic enzymes of Micrococcus halodenitrificans and Pseudomonas salinaria, and by Sierra and Gibbons (30). Sodium activated transport systems have been related to the requirement for the sodium ion by Drapeau et al (9).

There have been few references to works concerning both psychrophilism and halophilism as concurrent environmental influences although Gibbons and Payne (12) have considered possible dual effects as have Madeley et al (23) in their discussion of cell lysis as related to both temperature and salt concentration. Oppenheimer and Drost-Hansen (27) discussed multiple temperature optima in terms of water activity, a phenomenon in close relation to salt concentration. A review by MacLeod (22) gives perhaps the best treatment of the dual effects of temperature and salt concentration.

Investigations dealing with the isolation and characterization of Antarctic microorganisms have been reviewed by Sieburth (29) where it can be seen that earlier investigators failed to consider the psychrophilic character of the soil microflora. The works of Boyd and Boyd (5) and Boyd, Staley, and Boyd (6) in the McMurdo area demonstrate this approach since they utilized short time periods, the pour plate technique, and standard laboratory media which precluded successful psychrophilic isolations. However,

a modest beginning in understanding psychrophilic microorganisms in Antarctica has been made by Straka and Stokes (31) and Farrell and Rose (10). Meyer (25) utilized media which were more favorable to the growth of soil microorganisms than that used by Boyd and others, but his descriptive paucity has made repetition of his results difficult. Few of these investigators have considered the combined effects of the ecological pressures of low temperature and salt concentration.

Current work by Cameron and has associates (8) and by Benoit and Hall (unpublished data) has shown the value of specific techniques tailored to isolation of organisms from the singular conditions of the southernmost continent. Among these techniques are the use of precooled media, long incubation periods at low temperatures, spread plate methods, and soil enrichment media tailored to the region. It is to be expected that further modification of media and technique will result in a much more complete view of the Antarctic microflora.

MATERIALS AND METHODS

All bacterial isolates in this study originated from soil samples of the Dry Valley region, McMurdo Sound, Antarctica which were obtained during the austral summer from October to December 1967. The meanings for psychrophilism and halophilism are those as defined in the literature review with two exceptions. The reference to 0 C made by Ingraham for psychrophilism is raised to 2 C, and the minimum requirement for sodium in halophilism is 36 ppm. Media used in the isolation procedure are listed in Table I.

Chemicals used were of reagent grade and were obtained from the following manufacturers: Na_2SO_4, NaCl, and $CaCO_3$ were 'Baker Analyzed' reagents; Bacto-Agar, Dextrose, Gelatin, Lactose, Maltose, Peptone, Proteose Peptone #2, Tryptic Soy Broth, and Yeast Extract were Difco products. Soil extract was prepared by autoclaving one kg of Taylor Valley soil in one liter of distilled water with 0.5 gm $CaCO_3$ at 121 C and 15 lb pressure for 15 minutes. Soil used in preparation of the extract was obtained from a site near the southeast end of Lake Bonney about 75 meters from its edge in the vicinity of the hut. Particulate matter was removed by filtration through #1 Whatman filter paper, and the filtered extract was stored at 2 C until needed. In order to facilitate

Table I. Media used in the halophilic studies.

Constituents		Media Codes M12*	M14	M23	M24
Peptone	(gm)	5.0	-	5.0	5.0
Yeast Ext	(gm)	1.0	-	1.0	1.0
Tryptic Soy	(gm)	-	15.0	-	-
Agar	(gm)	15.0	15.0	15.0	15.0
Gelatin	(gm)	-	-	-	-
Soil Ext	(ml)	200	200	200	200
Don Juan HoH	(ml)	-	-	100	50
Ion Free HoH	(ml)	800	800	700	750
Final pH		6.4	7.2	5.1	5.4

*Final pH's of M-12 with additions of 5, 10, and 15 per cent sodium chloride were 6.2, 6.1, and 6.0 respectively.

transport to Blacksburg, some of the clarified soil extract was concentrated by evaporation until it was 25% of its original volume. This preparation was used in all subsequent studies in Blacksburg. Chemical analysis of the soil extract is given in Table II.

All media were prepared with ion free water obtained from a Barnstead demineralizer equipped with a #0802 standard cartridge. Glassware was washed by hand in Antarctica using Alconox detergent (Alconox, Inc.). All glassware was given ten final rinses, six of which were with distilled water and four with ion free water. Glassware at V.P.I. was cleaned in a Heinicke dishwasher (Heinicke Instruments Co.) with Heikol detergent (Heinicke Instruments Co.). The washer was programmed for a 20 second distilled water rinse after which there were four ion free rinsings by hand. Media were sterilized by autoclaving at 121 C and 15 lb pressure for 15 minutes.

Data concerning the locations of sample sites, the conditions under which the areas were sampled, and their V.P.I. soil index numbers are listed in Table III. Samples were taken in the field using sterile 50 ml screw capped glass vials. Sterile wooden spatulas were used to manipulate the soil, and care was taken to avoid contamination by taking all samples from a downwind, low profiled position. Soil samples were kept frozen during transit from the field to the laboratory at McMurdo. During transport

Table II. Analysis of Taylor Valley Soil Extract (Average of three samples)[1]

Ion	Concentration ppm
Ca	2.0
Mg	0.85
Na	36.0
K	1.7
OH	0
CO_3	0
HCO_3	32.0
SO_4	20.0
Cl	32.0
NO_3	2.7
PO_4	0.3
Mn	0
B	0
NO_2	.02
NH_4	0
Zn	.01
Cu	0
Co	0
Mo	0
Fe	.07
Al	0

[1]Personal communication from Roy E. Cameron, J.P.I., Pasadena, California. Analysis by Edward S. Babcock and Sons.

Table III. Index of sample sites[1]

V.P.I. Index Number	Date of Sample	Location	
391	12 Oct. 1968	Wright Valley	At edge of moraine west end of Don Juan pond
511	2 Nov. 1967	Taylor Valley	Alkaline area near Suess Glacier. Area received drainage from NW
546	22 Nov. 1967	Taylor Valley	Southwest of Suess Glacier in middle of an alkaline depression 15 m side. Sample of 3" depth
549	22 Nov. 1967	Taylor Valley	Same as 546 but near edge of alkaline area. 3" depth.
DJ1	6 Dec. 1967	Wright Valley	Don Juan pond 6 m from SW edge
DJ2	6 Dec. 1967	Wright Valley	Don Juan pond 20 m from SW edge
DJ5	6 Dec. 1967	Wright Valley	West end of Don Juan pond 30 m from moraine in dry sand of middle stream
DJ6	6 Dec. 1967	Wright Valley	3 m from DJ5 with coarse underlayer of salt

[1]The index of samples from similar or identical areas not included in this thesis is in the appendix.

from McMurdo to V. P. I. the soil samples were kept frozen in styrofoam containers packed with dry ice. Samples were stored in both laboratories at - 15 C.

Samples were processed at McMurdo in a small transfer room which was irradiated between experiments by means of ultraviolet lamps mounted on the ceiling and beneath the bench. At. V. P. I. all samples were processed in a Lab Con Co hood that could also be irradiated between experiments. The soil microflora were determined by weighing one gram quantities of soil on sterile aluminum weighing pans and then distributing these samples evenly over the surfaces of the appropriate media, or the soil was added to a sterile dilution blank. The blanks were prepared from ion free water augmented with 10% soil extract or NaCl in concentrations corresponding to those of the isolation media. Aliquots of 1:4, 1:40, and 1:400 dilutions were placed on the surfaces of precooled Petri plates of media in duplicate. A glass spreading rod was sterilized by repeated immersion in absolute ethanol followed by burning the ethanol on the rod; the sterile rod, when cooled, was then used to distribute the aliquots evenly over the media. All media, dilution blanks, and weighing pans were precooled to 2 C before processing. Petri plates were incubated at 2 C, 5 C, or 15 C for four, three, and two weeks, respectively, at which time they were counted on a Quebec colony counter. Negative plates

were retained for six weeks before they were discarded. Because of the long incubation periods in the low humidity of Antarctica and the high salt concentrations employed, the incubators were equipped with wick stoppered water bottles to increase the humidity. Isolates were selected from these plates on the basis of their Gram stain, colonial morphology, and pigmentation, and each culture was streaked several times to insure purity. Pure cultures were transferred to agar slants in screw capped glass tubes. The media corresponded to those on which the cultures were originally isolated.

The effects of growth factors, salt concentration, and incubation temperature were determined on selected isolates which appeared to be typical representatives of the halophilic microflora. They were grown in shake culture conditions in a Brunswick 'Psychrotherm' incubator at 180 rpm. Variations of medium M-12 were dispensed in 100 ml aliquots into cotton stoppered 250 ml Erlenmeyer flasks which were sterilized by autoclaving at 121 C and 15 lb pressure for 15 minutes. Since growth was not initiated at lesser concentrations, a 5 per cent inoculum was used in all liquid culture studies. Cells for the inoculum were taken from 72 hr liquid cultures in medium M-12 to which 5 per cent (w/v) sodium chloride had been added. Because earlier studies had indicated an increasing loss of viability during washing with the medium to be inoculated, 10 per cent soil extract, physiological saline,

or distilled water, this step and centrifugation were omitted unless otherwise indicated.

When inocula were washed, the following procedure was utilized. The cells were centrifuged at 20, 000 X G for 5 minutes at 10 C. They were washed in the medium into which they were to be transferred and were recentrifuged and suspended in the same medium at an O. D. of 0. 6. This adjustment was made on a Klett-Summerson Colorimeter with a red (640 mμ) filter. Growth was measured turbidimetrically by the same method for two random isolates, numbers H-25 and H-28 on variations of medium M-12. These variations included different salt concentrations and the addition of glucose.

RESULTS

Taylor Valley: Preliminary Results

Saline soils from Taylor Valley previously suspected of having a low biological content were added directly in one gram quantities to the surface of medium M-12 containing 5, 10, and 15 per cent added sodium chloride (w/v) and were incubated at 2 C, 5 C, and 15 C. Accurate colony counts could not be made by such direct plating because each soil particle served as a nucleus for growth of many bacteria; hence only qualitative results were obtained as indicated in Table IV. In the case of incubation at 2 C, four weeks were required to detect growth, whereas at 5 C and 15 C only two weeks were required. The size of the zones of growth observed around soil nuclei at 5 C and 15 C were similar; therefore, the 5 C treatment was discontinued in several future experiments.

These preliminary results indicated the presence of psychrophilic halophiles in numbers large enough to warrant the application of a dilution plating method. Excessive growth at 5 C and 15 C suggested, however, that the mesophilic and facultatively psychrophilic populations of these soils would require higher dilutions to obtain quantitative counts than would be the case for obligate psychrophiles.

Table IV. Qualitative effect of temperature and salt concentration on bacterial growth from Taylor Valley soil samples. [1]

Salt Concentration	Temperature		
	2 C	5 C	15 C
5%	+[2]	+	+
10%	0	+	+
15%	0	+	+

[1] One gram samples were plated directly on the surface of M-12 agar.

[2] + = appearances of colonies; 0 = negative after six weeks incubation.

Taylor Valley: Plate Counts

Samples from the saline areas of Taylor Valley were then plated on medium M-12 containing various concentrations of sodium chloride; the samples had been taken on 22 November 1967 before the summer soil temperature rose above 0 C and were kept frozen at -15 C for three days before plating. The results are shown in Table V. The highest counts were observed with 5 per cent added sodium chloride at 15 C (1.6×10^5 and 2.0×10^5 colonies per g.). At 5 C, the highest counts were obtained with no added sodium chloride and with 5 per cent added sodium chloride. The greatest disparity in counts between the 5 C and 15 C incubation temperatures was a 13-fold difference; however, in general only small differences were encountered, which supported the previous decision to omit the 5 C incubation temperature in certain experiments (See "Preliminary Results").

At 2 C the highest counts were also obtained with no added sodium chloride and with 5 per cent added sodium chloride; in fact, with 15 per cent and 20 percent concentrations of this salt, no colonies were observed even after six weeks. Furthermore, when these negative plates were subsequently incubated at 15 C, colonies appeared in numbers similar to those obtained on plates which had been incubated continuously at 15 C. No filamentous fungi or actinomycetes were observed in any case at 2 C, nor were pigmented

Table V. Halophilic counts per gram of Taylor Valley saline soil.

Media Used	Incubation Temperature (C^{o})	Colonies per Gram of Soil	
		Sample 546	Sample 549
M-12 No NaCl	15	2.5×10^4	7.2×10^4
	5	2.5×10^4	1.3×10^4
	2	1.4×10^3	5.1×10^3
M-12 5% NaCl	15	1.6×10^5	2.0×10^5
	5	1.2×10^4	1.2×10^5
	2	1.2×10^3	1.2×10^3
M-12 15% NaCl	15	1.2×10^3	1.4×10^3
	5	<40	<40
	2	<40	<40
M-12 20% NaCl	15, 5, 2	<40	<40

colonies at 5 per cent sodium chloride. The organisms found were largely Gram negative rods.

Wright Valley (Don Juan pond area) Preliminary Results:

Meyer et al (25) examined this pond in 1961 and predicted that the microflora would be unique. The pond water has a high soluble salt concentration (31% w/w), and the water temperature is below zero centigrade all of the year except during brief periods of the austral summer. Undoubtedly, this is the most saline area in the Dry Valley system and, therefore, a choice sampling location. Soil samples from areas surrounding the pond were taken in January 1967 after the soil temperatures were above freezing and were kept frozen at -15 C for six months before plating. The plating medium used was M-14. It will be recalled that in work dealing with Taylor Valley medium M-12 was used. Medium M-12 was not initially used in the study of this area as it was developed subsequent to the collection of these samples.

Soils from the northwest edge of the lake (Figure 1) were abiotic on medium M-14 while numerous bacteria were observed at sites taken farther from the lake in the vicinity of algal colonies visible in the stream beds. These streams fed the landlocked pond from the west. Counts were zero at both 2 C and 20 C at the former location, but at the latter site counts were 1.2×10^4

at 2 C and 8.3 x 10^5 bacteria per gram of soil at 20 C. The abiotic areas on the northwest side received little or no water as evidenced by the lack of incoming streams from glacial sources or winter snow. In the area of the glacial moraine and around the pond to the south and southeast counts ranged from less than 40 bacteria per gram of soil to 5.5 x 10^5 bacteria per gram at 2 C and 20 C on medium M-14.

Additional soil samples taken from the Don Juan pond area on 12 October 1967 were processed within two days and were plated in one gram quantities because of expected low counts on halophilic media. At the time of sampling the air temperature was -19 C and that of the unfrozen water was -24 C; the soil temperature was comparatively low. By this time medium M-12 had been shown to be superior to M-14, consequently, medium M-12 was hereafter used as the basal medium, not only for the present halophilic experiments but also in all other studies. In this work the following modifications of medium M-12 were employed: M-23, M-24, and M-12 enriched with 5 and 10 per cent sodium sulfate. With respect to M-23 and M-24, Don Juan water was added for enrichment to simulate the conditions of the pond area as closely as possible. For this same reason the pH was left at low levels since the pH of the pond water was 5.1.

The enrichment of medium M-12 with sodium sulfate was

based on the presence of high amounts of the sulfate ion as indicated by soil analysis.

The qualitative results are presented in Table VI; again it must be emphasized that accurate counts are not possible with the direct soil plating technique. It can be seen that moderate growth occurred around soil particles on the Don Juan water enriched media at 15 C, but there was almost no visible growth on those plates incubated at 2 C. Further, on media M-23 and M-24 at 15 C there was a difference only in the amount of growth exhibited. Medium M-23 (10 per cent Don Juan water) had smaller colonies localized around soil nuclei than did M-24. At the lower incubation temperature (2 C), colonies around soil nuclei could be seen only with difficulty, but their presence was shown by means of the Gram stain. With regard to medium M-12 enriched with 10 per cent sodium sulfate, there was no growth either at 2 C or 15 C; there was no growth on M-12 enriched with 5 per cent sodium sulfate at 2 C, but slight growth developed at 15 C. Gram stains from the latter showed Gram negative rods and cocci; in contrast, Gram stains of the growth from the Don Juan water enriched media exhibited Gram positive and negative rods and cocci. Moreover, the types of colonies observed on the 5 per cent sodium sulfate medium consisted mainly of small circular, raised, opaque and slightly tan colonies; whereas, a greater variety of colonial types were

Table VI. Qualitative results of direct platings from Wright Valley (Don Juan pond area)[1]

Temperature	Media Used			
	M23	M24	M12 5% Na_2SO_4	M12 10% Na_2SO_4
15 C	++	+++	++	0
2 C	trace	trace	0	0

[1]Incubation time 5-6 weeks.

observed on the Don Juan water enriched media. There were no actinomycetes observed on these media based on the absence of circular, conical, dry, and leathery colonies. Since quantitative data were not obtained from the above samples, they should be reexamined using a dilution plating technique.

Wright Valley (Don Juan Pond Area) Plate Counts:

Another set of samples was taken on 6 December 1967 when the ambient temperatures were 0 C and +8 C for air and water, respectively; those for the soils surrounding the pond were between these limits. The samples were processed after three months of storate at -15 C. On the basis of preliminary studies cited in the previous section, it had appeared that M-12 supplemented with sodium sulfate did not exhibit good growth supporting characteristics, consequently, sodium chloride was used in preparation of the halophilic media. Soil dilutions were plated on medium M-12 with various concentrations of sodium chloride and also media M-23 and M-24; the results are reported in Table VII. Sample sites are indicated in Figure 1. The colony counts observed on medium M-12 without added sodium chloride were in general agreement with those previously obtained on M-14 in January 1967. (See: Wright Valley, Don Juan pond area, preliminary results) Furthermore, sites 1, 2, 5, and 6 (see Figure 1) used in the present

Table VII. Halophilic counts per gram of soil from Don Juan Pond.

Media Used	Sample Numbers			
	DJ1	DJ2	DJ5	DJ6
		at 15 C		
M-12	1.7×10^2	1.2×10^3	1.1×10^5	2.3×10^4
M-12 5% NaCl	<40	<40	2.5×10^4	7.4×10^3
M-12 15% NaCl	<40	<40	<40	<40
M-23	<40	<40	1.2×10^3	1.3×10^3
M-24	<40	<40	1.2×10^3	5.9×10^3
		at 2 C		
M-12	<40	<40	2.2×10^3	<40
M-12 5% NaCl	<40	<40	1.2×10^3	<40
M-12 15% NaCl	<40	<40	<40	<40
M-23	<40	<40	<40	<40
M-24	<40	<40	48	<40

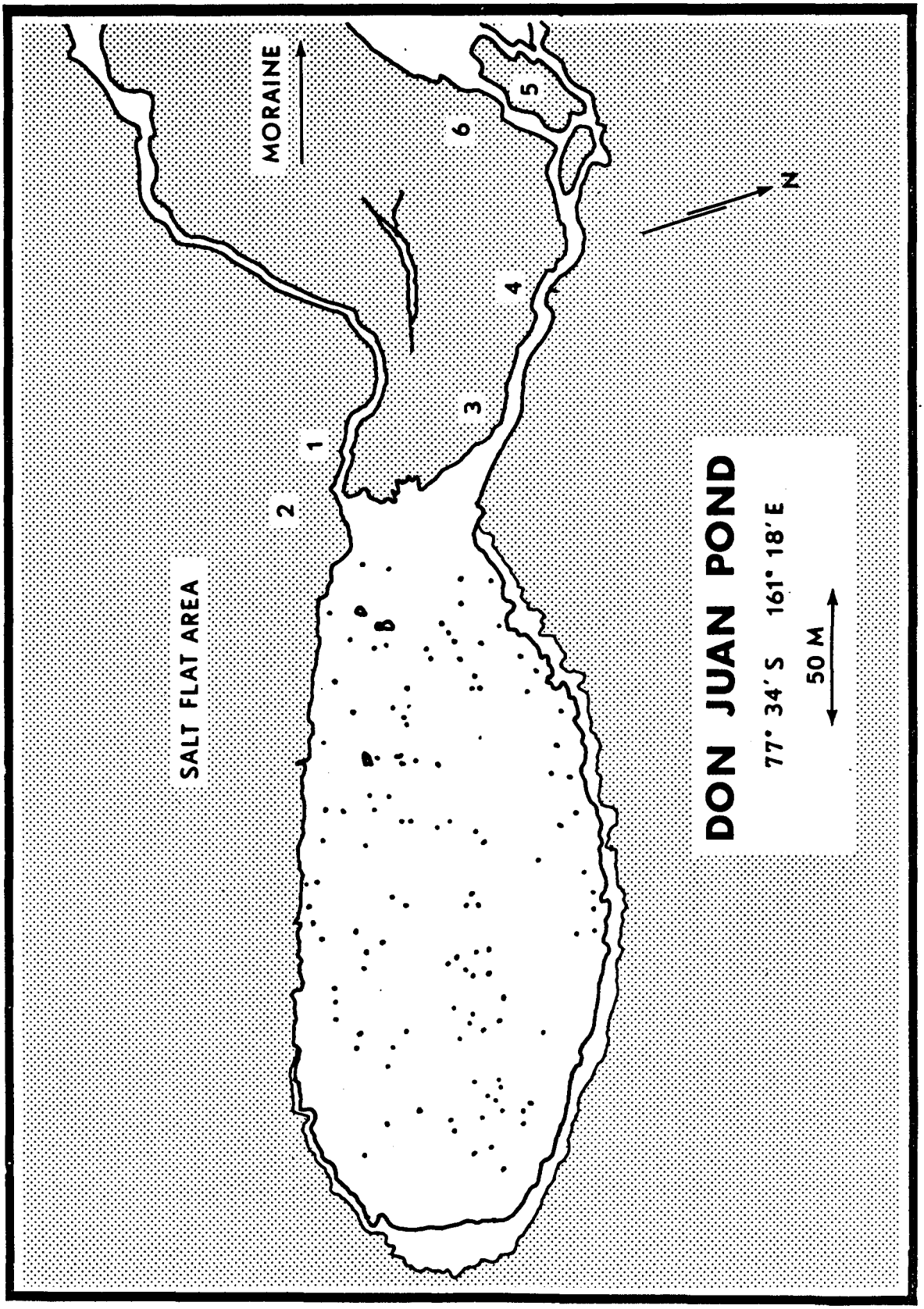
MORAINE
SALT FLAT AREA
1
2
3
4
5
6
N
DON JUAN POND
77° 34' S 161° 18' E
50 M

Figure 1

study were in the same general area as those samples taken in January 1967. More specifically, colony counts from the December 1967 sites 1 and 2 using medium M-12 were 1.7×10^2 and 1.2×10^3 at 15 C as compared to the January 1967 counts of 2.4×10^3 at 2 C and 5.3×10^5 at 20 C using medium M-14. In both instances the isolates were a variety of Gram positive and negative rods and cocci. It can be seen from Table VII that no growth occurred on samples from sites 1 and 2 at 15 C on media supplemented either with sodium chloride or Don Juan water. This was in spite of the fact that these two samples were from a region where there was a surface accumulation of salts. Since growth occurred only on unsupplemented medium M-12, the data indicate that the soil microflora from sites 1 and 2 was not salt tolerant.

In contrast, at sites 5 and 6 a significantly higher mesophilic bacterial population was observed at 15 C on medium M-12 than was found for sites 1 or 2; moreover, relatively high counts were obtained on the M-12 medium enriched with 5 per cent sodium chloride. The counts were 1/4 to 1/3 lower than those on unsupplemented M-12. No growth was observed on M-12 medium with 15 per cent added sodium chloride.

At 2 C there was no growth from sites 1, 2, or 6 on any of the media used in the December 1967 experiment; in fact, psychrophilic growth was observed only from soil samples at site number

5. In this case there were 1200 psychrophile colonies per gram of soil on medium M-12 enriched with 5 per cent sodium chloride as compared to 2200 per gram on medium M-12 from which this salt had been omitted. Counts averaging only 48 colonies per gram of soil were obtained on M-24 while medium M-23 exhibited no growth. The reduction of counts at 2 C compared to 15 C is in general agreement with the data previously determined for Taylor Valley (see Table V). However, with respect to sodium chloride concentrations, Taylor Valley counts at 15 C were highest in M-12 enriched with 5 per cent sodium chloride. In the present study of the Don Juan pond area the highest counts were obtained with unsupplemented medium M-12.

Bacterial isolates from sites 5 and 6 of the Don Juan pond area consisted largely of yellow, orange, red, and pink pigmented colonies, which were typical of those isolated from soils around fresh water ponds in other areas of the Dry Valley region (in both instances this characteristic growth was on unsupplemented medium M-12). However, fewer pigmented colonies were observed from sites 1 and 2. In contrast, colonies growing on medium M-12 supplemented with 5 per cent sodium chloride showed a characteristic distribution of only yellow and orange pigments. Although there was no growth of bacteria on media containing 15 per cent sodium chloride at 15 C from the Don Juan area (Table VII), at the Taylor

Valley sites there was growth under these conditions, notable in the fact that the colonies were nonpigmented. Growth on media M-23 and M-24 was restricted almost entirely to actinomycetes and yeasts.

Soil Samples from Beacon Valley:

Soil samples from a saline depression in Beacon Valley in which a 3-5 cm surface salt deposit had accumulated were abiotic on unsupplemented M-12 and medium M-12 with 5 and 15 per cent added sodium chloride. Nearby non-saline soils tested with unsupplemented medium M-12 were also generally abiotic.

Preliminary Testing of Growth and Nutrition:

Preliminary testing of growth patterns and nutrition was performed with two randomly chosen motile, nonpigmented Gram negative rods from Taylor Valley. The two strains were originally isolated on medium M-12 with 15 per cent added sodium chloride and were designated as H-25 and H-28.

Using fluid medium M-12 supplemented with 0, 5, 10, and 15 per cent sodium chloride, with shaking at 15 C, the optimal growth (most rapid) response for both isolates occurred with 5 per cent added sodium chloride. When fluid M-12 with 5 per cent supplemental sodium chloride was employed in shake culture at 15 C, it was found that the growth response of both strains was not affected by

omission of peptone, even after two transfers. Strain H-28 was tested further by altering the concentration of yeast extract. In the presence of the full amount of peptone (0. 5 per cent), the growth response decreased as the concentration of yeast extract was lowered from its initial value of 0. 1 per cent until no growth response was detectable at 0. 000 per cent. In the absence of peptone, no detectable growth response occurred at concentrations as high as 0. 003 per cent. Although neither of these organisms would grow at 20 C, static or shake, using M-12 medium with 5 per cent added salt, both were reinoculated several times. When subsequently transferred to 15 C with no further inoculation, both cultures grew. At 2 C in static culture, no growth was evident after eight weeks. It was not possible at that time to test cultures at 2 C under shaken conditions.

A preliminary growth experiment run on H-28, maintained in stock culture in M-12 medium with 5 per cent added sodium chloride, has indicated that at 15 C there is little difference in the final amount of growth at salt concentrations up to and including 15 per cent sodium chloride. There is, however, a great difference in the lag periods and the times required to reach maximum optical density. For example, H-28 on shake culture at 180 rpm reached a maximum O. D. of 0. 6, but the times required to reach an equivalent crop at 5, 10, and 15 per cent sodium chloride were 56 hours, 80 hours

and four weeks. The inoculum for the experiment was taken from a 72 hr M-12 culture with 5 per cent sodium chloride at 15 C.

Preliminary data indicated that the two organisms differed in their growth response in the presence of glucose using medium M-12 with 5 per cent added sodium chloride. Strain H-25 produced a turbidity of 134 Klett units in the absence of glucose but 455 Klett units in the presence of glucose. In contrast, strain H-28 was inhibited by glucose: H-28 produced a turbidity of 200 Klett units without glucose but only 50 Klett units with glucose. The stimulating effect of glucose for strain H-25 was less pronounced at higher salt concentrations. These results are indicated in Table VIII.

Both isolates were found to grow in liquid medium M-12 on shake culture at 15 C, although neither would grow when soil extract was not included in the medium. Further, both isolates evidenced slight growth at 17.5 per cent sodium chloride, and neither would grow with 20 per cent added salt.

Table VIII. The effect of glucose and sodium chloride on the growth response[1] of strain H-25 and H-28.

Additional Sodium Chloride	Without Glucose	With 1% Glucose
	Strain H-25	
5%	134	455
10%	130	373
15%	127	186
	Strain H-28	
5%	200	50
10%	197	49
15%	183	44

[1] Measured turbidimetrically in Klett units at 640 mμ.

DISCUSSION

Comparison of Results of the Sample Locations:

The data indicate that Taylor Valley and Don Juan pond contained dissimilar microbial populations although as samples were taken farther from the immediate area of Don Juan the results became more like those of Taylor. The differences in population were expressed in two ways, first in the number of psychrophilic colonies obtained under varying halophilic conditions and secondly the degree of salt tolerance expressed by a population. Concerning the number of psychrophiles (growth at 2 C) at a given location compared to the mesophilic population, it can be seen from the data that if the populations evidenced are assumed to be 2.5×10^4 and 7.2×10^4 at 0 per cent added NaCl (including both psychrophilic and mesophilic colonies), then the psychrophiles would form about 1/13 of this population. At 5 per cent added sodium chloride, psychrophiles compose 1/133 and 1/66 of the population. At 15 per cent added sodium chloride there were no bacteria able to express psychrophilic growth although the mesophilic population was considerable. This does not mean that there were no bacteria capable of growing either at 2 C or at 15 per cent added sodium chloride, but that none could express growth with this combination of temperature and salt.

In contrast, there were psychrophilic colonies from Don Juan pond only at one location and here the highest fraction of the mesophilic population was at 0 per cent added salt, not 5 per cent as was the case in Taylor Valley. One must assume further, that at Don Juan pond there are no psychrophiles in the immediate pond area able to survive the conditions there.

As regards the halophilic limits or halotolerance of the colonies that appeared from samples of both areas, Taylor Valley showed a more halotolerant population; in fact, the highest colony counts were at 5 per cent added sodium chloride implying that there is ecological pressure from these saline soils. There was also a portion of the population able to tolerant growth conditions of 15 per cent added sodium chloride at 15 C.

Again, in contrast, it appears that the population evidenced by plate counts of Don Juan pond did not have the halophilic tolerance of that at Taylor Valley at the temperatures used. Highest counts were from media containing minimal Na^+ (36 ppm), and there was no portion of the population able to tolerate 20 per cent added salt at 15 C or 2 C. Thus, there would appear to be little ecological pressure from an area of 31 per cent salt.

There were also differences between the two areas in colonial types as mentioned in the results; one of the most striking was the appearance of the actinomycetes which are mentioned later.

The populations cannot be adequately classified until further work is done on the individual physiological properties of isolates. These organisms must be tested for their response to different salt concentrations and growth temperatures, and such work must be done in pure culture. Reserve must be used when dealing with the characteristics of primary isolates.

Studies on Pure Cultures:

The two random isolates studied in terms of their growth on various concentrations of sodium chloride give a partial picture of that psychrophilic portion of the population isolated under the same conditions. As it was felt that these two gram negative rods typified the isolates, some inferences applicable to H-25 and H-28 may in turn apply to the entire group.

For the two isolates studied, maximal growth occurred with 5 per cent added sodium chloride at 15 C. They grew well with 15 per cent added salt at 15 C and only slightly with 17.5 per cent added salt. As indicated in the results, growth took place at the minimum level used (36 ppm) but not in the complete absence of sodium chloride meaning that these isolates were true obligate psychrophilic halophiles. This would indicate that these bacteria have a tolerance of salt concentrations over a fairly wide range from that amount of sodium contained in soil extract to 17.5 per

cent sodium chloride. Further, salt tolerance rather than stimulation is inferred as there was no significant difference in the final amount of growth as measured turbidimetrically between different salt concentrations although the salt concentration markedly affected the lag period. The lag increased from 24 hours to two weeks for 5 and 15 per cent sodium chloride respectively; the delay may have reflected an adaption to the media, or the growth of the culture at low salt concentrations may have permitted the selection of non-halophilic mutants. This latter case was possible as the inocula for all treatments were taken from a culture grown in medium M-12 with 5 per cent added sodium chloride transferred often over a period of several weeks. However, the fact that the final amount of growth was similar in the different salt concentrations implies that the long period is related to cellular adjustment of some physiological system rather than a mutation. As indicated in the results, further work must be done at other temperatures. Gibbons and Payne (12) studied the dual effects of salt concentrations and temperature finding that maximum growth of Halobacterium spp. was achieved at the optimal salt concentration, and that the effect of temperature was secondary. Also, growth was more rapid at one temperature while maximal cell yield was observed at another temperature. The data presented in this thesis are not in agreement with those of Gibbons and Payne. However, the disparity

could arise from the fact that Gibbons and Payne were using extreme or strict halophiles for their study. Also, the temperatures used by Gibbons and Payne were primarily in the mesophilic range (30 C to 50 C). It is possible that the salt tolerance of the Antarctic isolates would not be affected by salinity variations near the optimum temperature just as Halobacterium spp. would be near their minimum growth temperatures.

The observations of Madeley and others (23) concerning the lysis of a marine psychrophile by temperatures greater than the maximum growth temperature of the organism or low ionic strength appeared to be pertinent to H-25 and H-28. During routine microscopic assays for purity using the hanging drop method under darkfield conditions, it was noted that both Gram negative rods became swollen and lost their motility at 25 C. Also, the same occurred when the cells were suspended in salt solutions of 0.85 per cent or less. Although lysis was not observed, the cells appeared like those described by Madeley (23) and Abram and Gibbons (1), and it is likely that in both cases the cell walls were disrupted.

The Antarctic Desert as Related to Other Deserts:

Cameron (8) has reported some of the characteristics of Antarctic desert soils and selected desert soils of North America and Chile. The microorganisms found in the Antarctic desert soils

are related to temperate or tropical desert isolations with some noteworthy exceptions. Actinomycetes and fungi often compose a large part of any desert microflora, but this has not been found to be the case in Antarctica where the filamentous fungi are restricted to areas where moss is present or where there has been animal contamination (Benoit and Cameron, unpublished data). Actinomycetes have been found at very localized sites. When actinomycetes were detected by Benoit in Victoria Valley and at the Sulfur Cones on Ross Island, they were sometimes the only organisms found. In the area of Don Juan pond they composed a significant part of the population hereto undetected. Also unusual was the fact that they grew on acid media, contrary to the usual preference of these microorganisms for alkaline conditions. It must be concluded that the actinomycetes grow only under a very specific set of environmental conditions in the dry valleys, or that the isolation technique does not represent their true distribution.

Ecological Aspects:

In the Dry Valley region the ecology can be correlated with the microenvironment in relation to the limited microflora and the limited numbers of species. The results shown in Table V, indicate that even in the area of highest salt concentration the microflora required only minimal (36 ppm) amounts of salt, if any. Two models

might be used to explain some of the microbiology of Don Juan pond. First, there could be a continual influx of slightly halophilic bacteria from the less saline soils of the stream bed or from a water source in the Asgard Range during the austral summer. An alternate possibility is that the nonhalophilic bacteria could have been present in this area from a time when climatic conditions were more favorable for growth. It is known that some microorganisms survive under prolonged dormant conditions in the Dry Valleys (Benoit, unpublished data) which would partially explain the differences in incubation times necessary for growth under identical conditions of media and temperature with samples from different areas. It has been observed that the size of Don Juan pond varies considerably during the austral summer; it is largest in early spring and smallest in late summer. Since the pond is very shallow, the soil in the periphery of the pond is exposed to a variety of environments during the year. The variations in the soil microbiology may reflect this change in microclimate.

In contrast to Don Juan pond, Taylor Valley is a more diverse ecological system, in that there was no location that was landlocked and had as unusual composition as did Don Juan pond. However, the microbial populations in evidence there were under a restrictive environment as was the case at Don Juan pond. It may be postulated that the major difference between the ecologies of these two areas

lies in the fact that general conditions in Taylor Valley were more favorable to 'in situ' growth than they were at Don Juan pond. Specifically, there was no instance in Taylor Valley in which a possible toxic influence like Don Juan pond water was present (at a concentration of 31% w/w salt). This may have allowed growth of a halotolerant population in areas which were saline but had sufficient water. This was evidenced by the highest plate counts of a saline Taylor Valley soil occurring on medium M-12 with 5 per cent added sodium chloride. Also to be considered is the influence of the sea which was nearer to the Taylor Valley site. Zobell (35) demonstrated the transmission of bacteria of marine origin by inland breezes; the bacteria were demonstrated as 'marine' by their isolation from sodium chloride enriched media. Not to be forgotten is the possibility of the dormancy of a considerable portion of the Taylor Valley microbial population. The bacteria present could be a reflection of those microorganisms remaining from a time when the environment was favorable for their collective growth.

Evolutionary Aspects:

Psychrophiles have been found in Antarctic soils in great numbers not only by the V.P.I. group but by Cameron and his associates (8) and by Straka and Stokes (31). The psychrophile: mesophile ratio is greater in Antarctic soils as compared to temperate

soils which may reflect centuries of selection under severe climatic conditions favoring the psychrophile more than the mesophile. On the basis of the data obtained at Taylor and Wright Valleys, it has been observed that there is limited response of soil microorganisms to environmental pressure by halophilic conditions in soil systems. Henis and Eren (16) found that halophiles constitute no greater part of the population in saline soils than they do in nonsaline desert soils. Moderate to extreme halophiles may often be completely absent from Dry Valley soils containing high amounts of salts (Table VII), and they may appear in increasing numbers as the salt is diluted. Taylor Valley, however, showed a majority of bacteria able to grow on 5 per cent sodium chloride enriched M-12. In general, the soils of the Dry Valleys are saline according to Tedrow (32) and Cameron (8), and nonhalophilic or slightly halophilic bacteria are always found in higher numbers than moderate or extreme halophiles. It is well known that Halobacterium spp. and Photobacterium spp. are found in specific ecological niches in nature, but such microorganisms have not been found in these soil systems. Further data correlating the actual salt concentration of a given area is necessary.

Suggestions for Future Investigations:

Miers Valley and the Marble Point area should be included in

further halophilic studies because of their proximity to the sea. In addition Don Juan pond should be more extensively examined. This unusual niche provides a model for a microenvironment in which the effects of external forces can be noted and quantified. Future work should attempt to determine if the microorganisms of Don Juan pond are the result of an inflow of halophilic and nonhalophilic bacteria from the soil of the drainage basin above the pond, or if they may originate as aerial contaminants from the sea. Again, it must be considered that they may represent the remains of a microbial population when the pond was larger and did not have the high salt concentration it has today. Microorganisms were not detected in the pond water in the present study although Meyer et al (25) reported that three species were isolated. However, the data indicate that the pond water is a more adverse environment than the soil near or in the pond. Colonies of several microorganisms may be able to establish a more favorable environment on the surface of soil particles because the effects of high radiation, salinity, and cold temperature damage may be minimized. Possible synergistic nutritional effects in the soil may permit more rapid growth than is possible in mixed culture in a nonparticulate medium.

Future investigations of halophilic microorganisms in Dry Valley soils should utilize various modifications of medium M-12 in combination with different salts. It should be noted that medium

M-12 was not developed specifically for halophiles, and further modifications may be useful. Pure culture studies demonstrated that peptone could be eliminated from the medium for H-25 and H-28 without affecting their growth. In light of this, the quantity of peptone presently in M-12 may cause a masking of the growth of some halophiles: that is, these slow growing bacteria may be outgrown on the Petri plates by faster growing members of the soil microflora whose rapid growth might be attributed to the high peptone level. Further, peptone might even activate feedback or repression systems, which of course was not demonstrated by these isolates. A possible substitute for yeast extract, which could not be eliminated for two isolates tested, might be casamino acids.

Glucose inhibited the growth of isolate H-28 in M-12 and stimulated the growth of isolate H-25 dependent on salt concentration. However, these experiments must be repeated with more halophilic isolates before a conclusion can be reached as to the effect of carbohydrates on growth. Further work should include nutritional studies to determine which carbohydrates and amino acids can serve as carbon sources for pure cultures.

As mentioned above, sodium chloride and sodium sulfate were chosen as the major salts in the halophilic media, first because of the involvement of the sodium ion in halophilic metabolism and secondly because of the presence of large quantities of Na^+, Cl^-,

and $SO_4^=$ in Dry Valley soils as indicated by a soil analysis (Table II). Further studies should be done on the effect of other ions such as potassium, magnesium, and lithium in relation to a possible sodium requirement in these bacteria.

Psychrophilic salt tolerant bacteria isolated in this study were difficult to grow in pure culture on the original isolation medium although many of the isolates would grow at reduced salt concentrations. It was very difficult to obtain initial growth in liquid culture with some bacteria, and it was found necessary to use large amounts of the inoculum. The use of this large inoculum might explain how two successful serial transfers of H-25 and H-28 could be made in testing the extinction levels of peptone. It is obvious that until the nutritional aspects of the metabolism of the halophilic bacteria are more fully investigated, the physiology of these cells will remain obscure. Strain H-28 would grow under static conditions at 15 C, but the lag periods were as long as five weeks compared to several days for shake culture. It will be necessary to test the growth of such bacteria under shake and static conditions at a variety of temperatures in a variety of media with different salt concentrations to fully understand these phenomena. In simplest form these reactions may be due to a requirement for a given oxygen concentration in the medium. When such physiological characteristics are resolved, a more complete understanding of

the Antarctic microbial population will be possible, and physiological interactions within a given system can be studied.

Suggested Improvements in Methodology:

The long periods of incubation necessary for growth under conditions of high salt concentration, low humidity, and low temperatures necessitated procedures to prevent changes in the media. One such change was the alteration caused by desiccation. It was observed that the halophilic media began crystallizing after three weeks of incubation, even when measures were taken to increase the humidity within the incubators. Although the humidifying water bottles used to counteract this were effective, there was still some desiccation. It is suggested that the plates be placed in small numbers within sealed containers into which individual water bottles have been placed. This will be especially useful in incubators equipped with internal air circulating systems. Future experiments will require incubation periods lasting several months; therefore, a better control of the available water in culture media must be achieved.

As regards the plating technique, there are two points to be considered in subsequent experiments. First, because dilutions are being made from soils, a settling out of the soil particles and bacteria takes place at a sufficient rate to cause a gradient count,

even from one pipette. For this reason, the plates should be inoculated quickly after the dilution has been made. Secondly, halophilic media tend to absorb the aliquot very quickly; therefore, the plates should be immediately spread to attain even distribution of the microorganisms.

SUMMARY

Bacteria requiring both low temperature and sodium chloride for growth were isolated from the Antarctic polar desert. Colonies isolated from Taylor Valley soils grew at 2 C in media containing from 0 to 5 per cent added sodium chloride; at 15 C they were found to appear on media containing from 0 to 15 per cent added sodium chloride. Studies on two pure cultures indicate that the soils contain bacteria which can be classed as obligate psychrophilic halophiles. Until tested, the halophilic properties of the remaining isolates will be in question.

Contrasting results were obtained from the soil samples of Taylor and Wright Valleys. Although both areas were saline, there were differences in the halotolerance of the populations of each. Highest plate counts from samples of Taylor Valley were at 5 per cent added sodium chloride on medium M-12 whereas those from Wright Valley at Don Juan pond were at 0 per cent added sodium chloride. This would suggest that there had been response to environmental pressure by the bacteria from Taylor Valley saline soils while there was little or none by those bacteria in the Don Juan area. This can be partially explained on the basis of the toxicity of Don Juan water and the fact that the bacteria found there might have washed in from areas surrounding the pond.

Actinomycetes were isolated from the Don Juan pond area only if medium M-12 enriched with Don Juan water was used. These microorganisms were later found to be stimulated by this enrichment; they did not require the salts it contained. Don Juan pond proved to be a unique ecosystem for the study of environmental pressures on the microbial population.

It is postulated that moderate halophilism or a requirement for the sodium ion at a concentration of 36 ppm, is a survival advantage in the Dry Valley system, and that maximum growth may occur in concentrations up to 5 per cent added sodium chloride. However, at concentrations greater than this, bacteria are unable to grow under psychrophilic conditions.

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APPENDIX

Index of sample sites similar to those discussed in this thesis--Refer to Table III

V.P.I. Index Number	Date of Sample	Location	
547	22 Nov. 1967	Taylor Valley	Soil surface near 546
548	22 Nov. 1967	Taylor Valley	From area near 547 scraped from permafrost
550 551	22 Nov. 1967	Taylor Valley	Central part of an alkaline area east of Lake Bonney
552 553	22 Nov. 1967	Taylor Valley	Surface sample of middle of alkaline area at east end of Lake Bonney
DJ3 DJ4	6 Dec. 1967	Wright Valley	At Don Juan pond in area of streams between sample No's 1 and 5

ISOLATION OF PSYCHROPHILIC HALOPHILES FROM THE ANTARCTIC POLAR DESERT

Caleb Litteljohn Hall, Jr.

ABSTRACT

Saline soils in the Dry Valley region of McMurdo Sound, Antarctica, contained bacteria showing salt tolerance or requirement. Soils were plated by the spread plate method on soil extract-peptone-yeast extract media to which sodium chloride had been added in concentrations from 0 to 15 per cent (w/v). Bacteria isolated from these media at 2 C, 5 C, and 15 C were predominantly Gram negative rods with few Gram positive rods and cocci. No filamentous fungi or Halobacterium spp. were observed on the media used. At 15 C there were no isolates from media containing greater than 15 per cent added salt; however, counts of 1.4×10^3 colonies per gram of soil were found at this concentration. As the incubation temperatures were lowered, salt tolerance was lowered. The data indicate that the limited soil microflora observed in saline soils and ponds may be attributed to a combination of low maximal summer temperature and high salinity.