Mem. Fac. Fish., Kagoshima Univ.
Vol. 14, pp. 127～180 (1965).

Studies on the Marine Bacteria—II.

On the Specificity of Mineral Requirements of Marine Bacteria*

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Abstract

This study was undertaken to test the requirement of marine bacteria for inorganic salts, with an attempt to establish a reliable criterion to distinguish true marine bacteria from terrestrial ones.

In this paper the author deals with the results of morphological and physiological tests, with special emphasis on the requirement of inorganic salts in sea water, of several hundred strains of aerobic, heterotrophic bacteria isolted from various sources. Of the organisms used in this work, 275 cultures were isolated from sea water in the northern part of the North Pacific and the Bering Sea. In addition 13 cultures isolated from the surface of fishes, 7 strains from the National Collection of Marine Bacteria, 18 strains of *Vibrio parahaemolyticus* and similar strains, and 37 of other named strains of terrestrial bacteria were employed in the experiments.

The results obtained from comparative observations on the mineral requirements of marine and terrestrial bacteria were as follows.

1. These microorganisms showed different mineral requirements. This was demonstrated in experiments with a basal medium which contained only 0.05 per cent of polypeptone and 0.01 per cent of yeast extract as organic matter.

2. All the strains were tested for their growth capacity in the following five types of defined media during six days incubation at 25°C. The media (pH 7.8) contained, common to all, 0.05 per cent of polypeptone and 0.01 per cent of yeast extract, which were dissolved in; (a) pure water, (b) 0.5 per cent NaCl solution, (c) 3 per cent NaCl solution, (d) Herbst's artificial sea water diluted six-fold, and (e) Herbst's artificial sea water.

3. The results above mentioned tell us that one is able to group the test organisms into three patterns by growth capacity manifested in these media. One of them, which includes terrestrial bacteria, is characterized by the capacity to grow in five types of defined media. Another group lacks the capacity to grow in (a) medium or both (a) and (b) media. The last one, to which the majority of marine isolates belong, is characterized by incapability to grow in the media (a), (b), and (c). Each of the three types were designated as Terrestrial (T-) type, Halophilic (H-) type, and Marine (M-) type bacteria.

4. Terrestrial type bacteria showed a moderate growth without any supplement of inorganic salts to the basal medium and ones who did not take special requirements of mineral salts. Most of them grew best at 0.5 per cent of salt concentration, and tolerated some growth in 5 to 7 per cent NaCl. They could grow at 37°C.

5. Halophilic type bacteria were ones which required to NaCl, but did not require to the other minerals in sea water. They grew best in 3 per cent of salt concentration and tolerated some growth at 12 per cent NaCl. And about half of were able to grow in 37°C. Their NaCl requirement could be partialy replaced with other salts. In other words, *V. parahaemolyticus* only requires NaCl, and concerning the *V. parahaemolyticus*'s growth, NaCl has a function like osmolar control.

6. Marine type bacteria have special requirement for minerals. Not only NaCl but also

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* From a thesis submitted for the degree of Doctor of Fishery Science at the University of Hokkaido, June, 1965. A part of this work was published at the General Meeting of Japanese Society of Scientific Fisheries, Otaru, October, 1963.

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other minerals such as K-, Mg-, and Ca-salt in sea water are needed for their growth. Most of them were able to grow in the artificial sea water media with a salt concentration ranging from 0.5 to 12 per cent. In the majority of the strains, the salt concentration optimum for growth was found to be 3 to 7 per cent. But they could not grow at 37°C. They also grew in $NaNO_3$ or Na_2SO_4 medium under the existence of K-, Mg-, and Ca-salt. Na^+ was indispensable for their growth, and it could not be replaced with any other cations. They specially required Na^+ and the other salts in sea water contrasting with other types of bacteria. In other words, the sea water has a role of nutritional supply rather than that of osmotic regulation on the growth of Marine type bacteria.

7. Of 275 strains isolated from sea water in the northern part of the North Pacific and the Bering Sea, about 32 per cent were Marine type, about 50 per cent were Terrestrial type, and about 18 per cent were Halophilic type. It had been known that approximately 95 per cent of the bacteria occurring in the sea are Gram-negative. Most bacteria of Gram-negative flagellate rods belonged to the Marine type.

8. Although organisms belonging to these three groups have been found widely in the sea, the author believes that only the Marine type bacteria in three types should be designated "marine bacteria" in the strict sense. And other types of bacteria which occurred there were in the main composed of the survivors of the continental flora. The *V. parahaemolyticus* also did not belong to marine bacteria

9. True marine bacteria could best be distinguished from land contaminants present in sea water by their growth capacity manifested in the five types of defined media established.

10. The Marine type bacteria were shown to require a relatively high concentration of Na^+ for their optimal growth and metabolic activity, and also other inorganic salts such as K-, Mg-, and Ca-salt in sea water for their growth. In the investigation on the effects of NaCl and these other inorganic salts on the metabolism of intact cells of Marine type bacteria, it was revealed that NaCl had a specific, positive effect on their enzymic systems, while the other salts had cytological effects on their cell structure, particularly on the structure of cell walls, rather than the effect on their metabolic activity.

Contents

Introduction

Investigations into the microorganisms in the sea have started in the last century, but the earlier work was almost limited to the findings of abundance and distribution of bacteria and bacterial make-up in the sea. Microorganisms are regarded as an important group in marine communities, since they play a significant role in the formation and transformation of organic matter in the sea. They attack dead organic matter and regenerate it into inorganic matter which is indispensable for the growth of marine plants. In recent years marine microbiology has been extended to cover the studies on their function in the cycle of organic and inorganic matter in the sea, and it is becoming one of the essential part of oceanography, especially in the study of the productivity of the sea. The literature on marine bacteriology is adequately reviewed by ZoBell (1946) and Kriss (1963).

The sea is a specific environment for microbial life. Compared with terra and fresh-water, the sea as a habitat of microbes can be characterized by the high concentration of salts, relatively constant low temperature, general paucity of organic matter and great hydrostatic pressure in deep water. It is known that some land and fresh-water bacteria are remarkably tolerable to considerably high salinity. When those tolerable species are carried into the sea by agencies, such as rivers, sewage outfalls, wind, birds, and so on, they can survive long in the new habitant, appearing as common inhabitants in the sea, sometimes even in the off-shore region. However, these species should be defined as adventitious contaminants, differing from the truly marine species which are originally distributed in the sea. The term, marine bacteria, has been used rather ambiguously. Species occurring in the sea, both those originally inhabiting the sea and those of terrestrial origin, are called marine bacteria inclusively.

Korinek (1927) proposed that marine bacteria could be distinguished from non-

marine bacteria on the basis of their salt tolerance. However, some of the terrestrial and fresh-water bacteria are known to be tolerant to high salinity in culture, so that adventitious species cannot be effectively separated from true marine species by this method. ZoBell and Upham (1944) defined marine bacteria as those requiring a medium containing sea water at the initial stage of growth in culture. This method again can not be employed for the separation of adventitious species from marine forms, because some terrestrial and fresh-water species begin to grow in a saline medium. MacLeod and Onofrey (1954, 1956, 1957) mentioned that effectiveness of sea water in stimulating the growth of marine species are mainly due to the supply of inorganic ions derived from sea water. They found that such bacteria as those growing in a sea water media require specifically Na^+ and suggested that the requirement of Na^+ can be a point defining the nature of true marine bacteria. Halophilic bacteria have been isolated from fresh-water and soil, and these halophiles have been reported to have specific requirements for Na^+ (Larsen, 1962). It is evident that a specific need for Na^+ is not a characteristic unique for bacteria marine origin was previously imagined.

This study was undertaken to test the requirement of bacteria occurring in the sea for several kinds of salts in sea water, i. e., Na-, K-. Mg- and Ca-salt, with an attempt to establish a reliable criterion to distinguish true marine bacteria from halophilic bacteria originating from land or fresh-water. Several hundred strains of aerobic-heterotrophic bacteria isolated from sea water in the northern part of the North Pacific and the Bering Sea were tested. This paper deals with the results of morphological and physiological tests of those strains, with special reference to their mineral requirements.

Experiments

I. General character of test microorganisms

Several hundred strains of aerobic-heterotrophic bacteria isolated from various sources were studied morphologically and physiologically.

1. Source of materials, isolation of strains and pure culture derivation

During cruise 44 of the M. S. Oshoro Maru, Training Ship of the Faculty of Fisheries, Hokkaido University, in the northern part of the North Pacific and the Bering Sea during the period from June 8 to July 27, 1959, Mr. T. Kimura and Mr. T. Matsuura of that university carried out bacteriological samplings at 22 stations, by the instruction of Dr. M. Sakai, Professor of Microbiology. The samplings were made with sterile bacteriological J-Z water samplers (ZoBell, 1946) from 12 depths from the surface to about 1,000 meters of depth at each station (Fig. 1, Table 1). Immediately after the samplings, the water samples

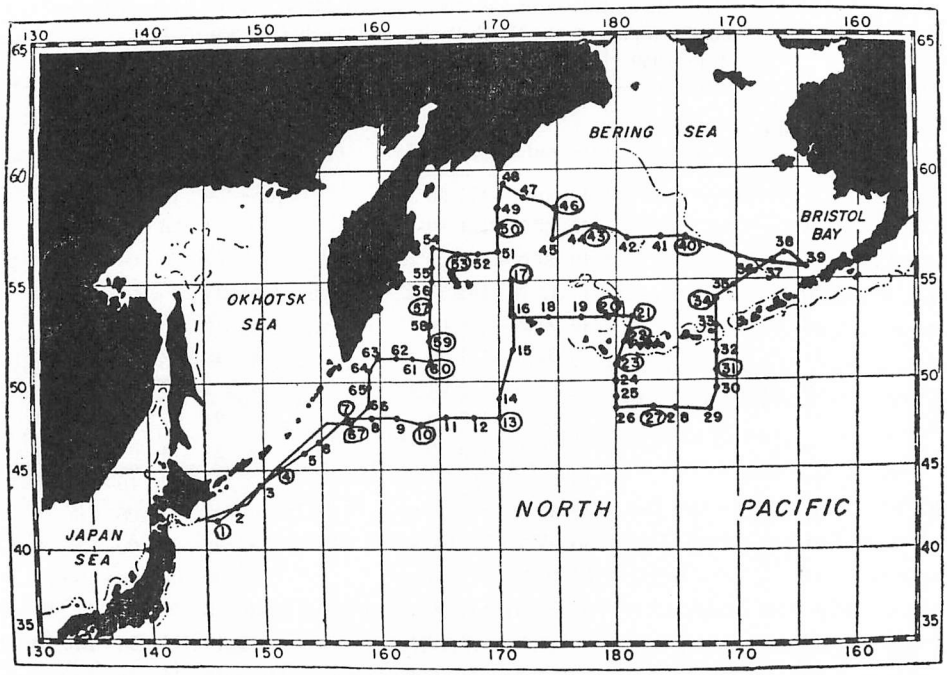


Fig. 1. Track and hydrographic stations in the "Oshoro Maru" cruise 44 to the northern part of the North Pacific and the Bering Sea in June-July, 1959
Circles: Microbiological stations
(Quoted from "Data Rec. Oceanogr. Expl. Fish. No. 4, 1960, Faculty of Fisheries, Hokkaido University")

were directly inoculated into Medium 2216 E (Morita *et al.*, 1955) in a laboratory aboard. The cultures were incubated at 25°C for 6 days, and then colonies grown on and in the agar plate were counted by Mr. Kimura and Mr. Matsuura. The cultures were then put into a refrigarator to bring back to land. At the university laboratory representatives of various colonies were transferred to agar slopes of the same composition. Two-hundreds seventy-five isolates thus obtained were placed at the author's disposal through the courtesy of Dr. Sakai. The strains were: 47 Gram-negative non-motile rods, 94 Gram-negative motile rods, 54 Gram-positive non-motile rods, 5 Gram-positive motile rods, and 75 Gram-positive cocci. These strains were numbered 1001-1 to 1253-6 according to the order of locality of collection.

In addition, 13 cultures isolated from fish bodies were used in this study. They were isolated from several species of fish caught in Kagoshima Bay in February, 1962. The isolation was made in the manner described by Shewan *et al.* (1960).

Seven strains of agar-digesting marine bacteria were provided for the author for this study from the National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland. They had been listed as NCMB No. 800, 803, 805, 820, 822, 823 and 825.

Table 1. Microbiological stations on the "Oshoro Maru" cruise 44 to the northern part of the North Pacific and the Bering Sea in June—July, 1959

Stations	Position	Date and time of sampling	
	Latitude Longitude	Date (in 1959)	Time
1	42°00′N—146°00′E	June 9	16 : 40—18 : 19
4	45°03′N—151°32′E	〃 11	08 : 30—10 : 30
7	48°00′N—156°59′E	〃 12	23 : 04—00 : 37
10	47°42′N—163°31′E	〃 14	16 : 05—18 : 10
13	48°00′N—170°00′E	〃 16	16 : 30—17 : 40
17	55°00′N—171°20′E	〃 19	12 : 43—14 : 00
20	53°16′N—179°04′E	〃 22	18 : 03—19 : 54
21	53°16′N—178°32′E	〃 24	17 : 35—18 : 31
22	52°01′N—179°30′E	〃 24	14 : 03—15 : 24
23	51°00′N—180°00′	〃 24	22 : 10—23 : 36
27	48°32′N—177°20′W	〃 26	17 : 08—18 : 30
31	50°25′N—171°59′W	〃 28	20 : 00—21 : 39
34	54°10′N—172°00′W	〃 30	09 : 00—10 : 28
40	57°01′N—174°30′W	July 8	15 : 56—17 : 05
43	57°38′N—177°59′E	〃 11	11 : 00—12 : 10
46	58°19′N—174°50′E	〃 13	14 : 12—15 : 27
50	57°19′N—169°58′E	〃 15	11 : 11—12 : 47
53	56°20′N—166°18′E	〃 16	19 : 31—20 : 49
57	53°54′N—164°16′E	〃 18	11 : 48—13 : 13
59	51°59′N—164°04′E	〃 19	11 : 20—12 : 57
60	51°02′N—164°18′E	〃 19	19 : 30—21 : 13
67	47°43′N—157°10′E	〃 23	07 : 53—09 : 00

(Quoted from "Data Rec. Oceanogr. Expl. Fish. No. 4, 1960, Faculty of Fisheries, Hokkaido University")

Named strains of terrestrial bacteria which were used for comparison with test bacteria are shown in Table 2. Of them *Vibrio parahaemolyticus* (FUJINO *et al.* 1951) SAKAZAKI *et al.* 1963, Pathogenic Halophiles, has been presumed by Japanese workers as one of causative bacterium of enteritis type food poisoning.

2. *Media and cultural conditions*

The microorganisms from marine origin were cultivated on slants of a modified Medium 2216 E at 25°C for 6 days. The medium was prepared with the following constituents: polypeptone (Daigo Eiyo Kagaku Co., Ltd., Japan), 5g; yeast extract (Daigo Eiyo Kagaku Co., Ltd., Japan), 1g; ferric phosphate, 0.01g; agar (Nissui Seiyaku Co., Ltd., Japan), 15g; and Herbst's artifical sea water, 1 liter; the pH was adjusted to 7.6–8.0; and then autoclaved at 15 lbs pressure.

The named strains of terrestrial bacteria, with the exception of *V. parahaemolyticus* and *Lactobacillus acidophilus*, were cultivated on nutrient agar slants. The

Table 2. Named strains used for comparative studies with marine bacteria

Organism	Source	Donor*
Pseudomonas aureofaciens IAM-1001		IAM
// *ovalis* IAM-1002	Soil	IAM
// *aeruginosa* IAM-1007	Pus from wounds	IAM
// *dacunhae* IAM-1089	Soil	IAM
// *schuylkilliensis* IAM-1154	Schuylkill River	IAM
// *trifolii* IAM-1309	Clover hay	IAM
// *saccharophila* IAM-1504	Stagnant water	IAM
// *putrefaciens* IAM-1509	Tainted butter	IAM
// *fluorescens* FHU-*Ps.* 2	Sewage	FHU
// *fluorescens* FHU-*Ps.* 3	Water	FHU
Achromobacter cycloclastes IAM-1013	Soil	IAM
// *liquidum* IAM-1667	Water	IAM
Proteus mirabilis NCTC*-6197	Putrid meat	AKU
// *mirabilis* SMLU*-398	Feces of infants with child diarrhoea	AKU
// *morganii* NCTC-235	Feces of infants with summer diarrhoea	AKU
// *morganii* SMLU-3373	Stool	AKU
// *vulgaris* NCTC-4175	Putrid meat	AKU
// *vulgaris* SMLU-854		AKU
// *rettgeri* NCTC-7475	Cholera-like epidemic among chickens	AKU
Bethesda ballerup	Feces of a woman from the town of Ballerup	FHU
Escherichia freundii	Canal water in Holland	FHU
// *coli* (0-26**)	Human feces	FHU
Bacillus mesentericus fuscus	Canned peas	FHU
// *mesentericus vulgatus*	Milk	FHU
// *megatherium* NRRL*B 739	Soil	FHU
// *subtilis* NRRL* 558	Soil	FHU
// *prodigiosus*	Soil	FHU
// *butyricus*	Milk	FHU
// *denitrificans*	Soil	FHU
Micrococcus lysodeikticus		FHU
// *aureus*	Pus in wounds	FHU
// *albus*	Pus in wounds	FHU
// *perflovus*	Pus in wounds	FHU
Sarcina lutea	Soil	FHU
Lactobacillus acidophilus	Feces of milk-fed infants	FHU
Vibrio metschnikovii IAM-1039	Fowl dead of a cholera-like disease	IAM
// *tyrogenus* IAM-1080	Cheese	IAM
// *paraheamolyticus* (0-1 to 0-12)***	Feces of the patients of raw fish meat poisoning	NIHJ

* IAM: Institute of Applied Microbiology, University of Tokyo, Japan;
FHU: Faculty of Fisheries, Hokkaido University, Hokkaido, Japan;
AKU: Faculty of Agriculture, Kyoto University, Kyoto, Japan;
NCTC: National Collection of Type Cultures, London, England;
SMLU: Strich School of Medicine, Loyola University, U.S.A;
NRRL: Northern Utilization Research Branch, U.S. Dept. of Agriculture, Peoria, U.S.A.;
NIHJ: National Institute of Health, Tokyo, Japan.

** Serotype

*** Serotype 0-1 to 0-12, total 12 strains

culture of *V. parahaemolyticus* was carried out on agar slants prepared by solidifying nutrient broth containing 3 per cent NaCl with 1.5 per cent agar. *L. acidphilus*, on the other hand, was cultivated in a milk medium. The stock cultures of organisms were maintained at laboratory temperature (15° to 20°C) renewing the culture media every three months. *L. acidophilus* alone was maintained by transfering it weekly to the milk medium.

3. *Locality of isolation and main characters of some selected marine isolates*

Morphological, cultural, and physiological characters of the organisms were tested employing the following techniques: All media were prepared using three solutions as a diluent. One of them, artificial sea water (full or half strength), was used for the strains of marine origin. Each 3 per cent and 0.5 per cent of NaCl solution were used for *V. parahaemolyticus* and terrestrial bacteria. Unless special mention is made, the cultures described in this paper were always incubated at 25°C.

Morphological and cultural characteristics

Organisms were stained by Hucker's modification of Gram's stain after incubation for 1 to 2 days on the nutrient agar slants. Their motility was examined on similar cultures by hanging-drop preparation. Motile ones alone were stained for flagella; the technique was based on a method employing Fisher and Conn's modification of Bailey's flagella stain. And handling cultures for strains of marine origin were maintained with 2 to 3 ml of sterile artificial sea water (half strength) (Manual of microbiological method, 1957).

Pigment. Strains had grown on nutrient agar at the optimal temperature for 2 days and were then kept at room temperature for a week in diffuse daylight. Pigment was recorded as yellow or orange.

Pyocyanin and fluorescein. Cultures were streaked on the two media (King A and B) recommended for maximum producing media of pyocyanin and fluorescescein by King (1954), and each of them was examined in daylight and ultraviolet light after an incubation period of 1, 3, and 6 days.

Luminescence. The test organisms were incubated on a medium composed of 1 per cent polypeptone, 0.2 per cent yeast extract, 0.3 per cent glycerine, and 1.5 per cent agar in artificial sea water, and the luminescence was examined on the culture with completely dark-adapted eyes after 2 days incubation.

Relation to oxygen. Duplicate tubes containing semisolid nutrient agar (0.3 per cent agar) were used, and test organisms were inoculated by stabbing. After inoculation one of the paired tubes was coverd with a layer of sterile melted petrolatum to a depth of 2 cm. Then the growths in the sealed and unsealed tubes were observed after 6 days.

Sensitivity to a 'vibriostatic' compound (0/129). The cultures were tested for sensitivity to a vibriostatic compound (2, 4-diamino 6, 7-di-*iso*propyl pteridine) as described by Shewan *et al.* (1954). Inhibition of growth after 24 to 48 hours was

tested using paper disks impregnated with the agent.

Growth at 37°C was observed in the nutrient broth for 6 days.

Physiological tests

Gelatin hydrolysis was carried out by adding using 0.5 per cent (W/V) gelatin in nutrient agar and flooding 5 to 6 days growth with acid mercuric chloride.

Action on litmus milk. The cultures were incubated in litmus milk medium for 6 days, and the changes in appearance were noted at definite intervals.

Hydrolysis of starch was tested on 6 day cultures grown in nutrient agar containing 0.2 per cent (W/V) soluble starch by flooding with iodine solution.

Indole formation was determined by Ehrlich-Bohme's reagent after incubation for 6 days in peptone water.

Hydrogen sulphide formation was detected by using strips of filter paper impregnated with lead acetate on cultures incubated for 6 days in nutrient broth.

Reduction of nitrate to nitrite. Nitrite was tested by the Griess-Ilosvay reagents after incubation for 6 days in peptone water containing 0.1 per cent (W/V) KNO_3. Powdered zinc was used to test false negatives.

Ammonia production was tested by Nessler's reagent after incubation for 6 days in peptone water.

Methylene blue reductase. One drop of 1 per cent (W/V) aqueous methylene blue was added to 5 ml broth culture, the tube was well mixed and incubated at 37°C for 2 hours. Complete decolorization below the top a half cm was recoreded as positive.

Voges-Proskauer test. Cultures were tested after incubation for 6 days in medium with (%, W/V): polypeptone, 0.5; glucose, 0.5; dipotassium phosphate, 0.5. In this procedure, the test reagent added to the culture was 0.3 per cent creatine in 40 per cent KOH solution.

Methyl-red test was read after 6 days incubation in glucose phosphate peptone medium.

Growth in Koser's citrate medium was read after incubation for 6 days. Strains which showed growth after twice serial subcultures were recored as positive (in Skerman, 1959).

Hydrolysis of urea. Cultures were incubated for 6 days on slants of urea agar medium (in Skerman, 1959). To ensure whether the alkalinity produced was due to urea hydrolysis or not, the urease-positive strains were inoculated in a control medium without urea. Only those strains which gave a markedly more alkaline reaction in the urea medium were considered to be true positives.

Catalase formation. Catalase was determined by addition of commercial 40 per cent hydrogen peroxide 1:20 (V/V) with pure water to agar slant cultures.

Oxidase test. Kovacs's (1956) method was used in cultures grown on solid medium for either 24 or 48 hours.

Glucose utilization. The methods of Hugh and Leifson (1953) and Leifson (1963) were used to determine whether glucose was used oxidatively or fermenta-

Table 3. Locality of isolation of some selected strains of bacteria from sea water

Strain	Locality of isolation		No. of strains of similar form found
	Station*	Depth in meters	
1007-1	1	98	10
1040-1	10	63	25
1055-1	13	694	20
1055-2	13	694	17
1064-2	17	670	11
1135-4	31	495	138
1179-2	46	56	27
1197-4	50	863	27

* See Table 1.

Table 4. Morphological and cultural characters of the selected strains

Strain	Cell-form	Flagella	Gram's stain	Pigments (water-insoluble)	Pyocyanin	Fluorescence (under U.V.)	Luminescence	Growth at 37°C	Relation to oxygen
1007-1	R	P	−	−	−	−	−	+	A
1040-1	R	M	−	−	−	+	−	−	A
1055-1	R	M	−	−	−	+	−	−	A
1055-2	SR	non	−	O	−	−	−	−	A
1064-2	SR	M	−	−	−	−	−	+	FAn
1153-4	R	P	−	−	−	−	−	+	FAn
1179-2	C	non	+	Y	−	−	−	±	FAn
1197-4	R	M	−	−	−	+	−	±	A
F-12	SR	M	−	B	−	−	+	−	FAn
V. parahaemolyticus	SR	M	−	−	−	−	−	+	FAn
V. metschnikovii	CR	M	−	−	−	±	−	+	FAn
Ps. aeruginosa	R	M or L	−	−	+	+	−	+	FAn
Ps. fluorescens	R	M	−	−	−	+	−	±	FAn
Pr. vulgaris	R	P	−	−	−	−	−	+	FAn
E. coli	R	P	−	−	−	−	−	+	FAn

Key: R, rods; SR, short rods; CR, curved rods; C, cocci; P, peritrichous; M, monotrichous; L, lophotrichous; O, orange; Y, yellow; B, buff; A, aerobic; FAn, faclutatively anaerobic; −, negative; ±, weak positive; +, positive.

Table 5. Physiological characters of the selected strains

Strain	Gelatin hydrolysis	Action on litmus milk	Starch hydrolysis	Indole production	H_2S production	Nitrate reduction	Ammonia production	V. P. test	M. R. test	Growth in Koser's medium	M.B. reductase	Urease	Catalase	Oxidase (Kovacs)	Hugh-Leifson test	Arginine dihydrolase	Sensitivity to 0/129
1007-1	−	NC	−	−	−	+	+	−	−	−	−	−	+	+	NC	−	−
1040-1	+	Al, P	+	−	+	−	+	−	−	−	+	−	+	+	O	−	−
1055-1	+	Al, P	+	−	+	−	+	−	−	−	+	−	+	+	O	−	−
1055-2	+	NC	−	−	−	−	+	−	−	−	+	−	+	−	Alk	−	−
1064-2	−	NC	−	−	±	−	−	−	−	−	−	−	+	+	NC	−	+
1135-4	+	NC	−	−	+	−	−	−	−	−	±	−	+	−	F	−	+
1179-2	−	NC	−	−	−	−	−	−	−	−	+	−	+	−	NC	−	+
1197-4	+	Al, P	+	−	+	−	+	−	−	−	+	−	+	+	O	−	−
F-12	−	NC	−	−	+	+	+	−	−	−	−	−	+	−	F	−	+
V. parahaemolyticus	+	Al, P	+	+	+	+	+	−	+	+	+	−	+	+	F	−	+
V. metschnikovii	+	A, C	+	+	−	+	+	+	±	−	−	−	+	−	F	+	+
Ps. aeruginosa	+	Al, P	−	−	−	+	±	−	−	+	±	±	+	+	O	+	−
Ps. fluorescens	+	Al	±	−	−	+	−	−	−	+	+	±	+	+	O	+	−
Pr. vulgaris	+	Al, P	±	+	+	+	+	−	±	−	+	+	+	−	F	−	−
E. coli	−	A, C	+	+	+	+	+	−	+	−	±	+	+	−	F	−	−

Key: −, negative; ±, weak positive; +, positive;
NC, no change; Al, alkaline; A, acid; C, coagulation; P, peptonization;
O, oxidative: F, fermentative; Alk, alkaline reaction in open tube.

tively. Controled tests without glucose were also done.

Arginine metabolism. The mode of action on arginine was studied by means of the semisolid medium of Thornley (1960).

Production of acid and gas from carbohydtates

Cultures were incubated for 6 days in a medium composed of (%, W/V): Bact-peptone, 0.02; dipotassium phosphate, 0.03; bromthymol blue, 0.003; agar, 0.3; test carbohydrate, 1.0. Each tube of the semisolid medium was inoculated by stabbing and was examined regularly for acid and gas prodution. The following carbohydrates were used: glucose, xylose, arabinose, rhamnose, galactose, fractose, sucrose, lactose, maltose, raffinose, glycerine, and mannit. Controled tests without carbohydrate were also done.

Details of results

The majority of the strains used in this study were subjected to morphological and physiological tests. The complete details on experimental results of all the test microorganisms are not described here. The experimental results of the test

Table 6. Utilization of carbohydrates of the selected strains

Strain	Production of acid and gas from**											
	Glucose	Xylose	Arabinose	Rhamnose	Galactose	Fractose	Sucrose	Lactose	Maltose	Raffinose	Glycerine	Mannit
1007-1	±	±	+	−	+	+	−	−	−	−	±	±
1040-1	+	*−	*−	*−	+	+	+	±	+	−	+	+
1055-1	+	*−	*−	*−	*−	+	+	*−	+	*−	*−	*−
1055-2	*−	−	*−	*−	*−	+	*−	−	*−	−	−	−
1064-2	±	+	−	−	+	+	±	±	+	±	−	+
1135-4	+	−	−	*−	−	−	±	−	−	±	−	−
1179-2	±	−	−	−	−	+	−	−	−	−	+	+
1197-4	+	*−	*−	*−	*−	*−	+	*−	+	*−	±	*−
F-12	+	−	−	−	+	+	*−	−	+	−	+	−
V. parahaemolyticus	+	*−	+	*−	+	+	*−	*−	+	*−	+	+
V. metschnikovii	+	−	*−	*−	+	+	±	+	+	+	+	+
Ps. aeruginosa	+	+	+	*−	+	±	*−	*−	−	*−	±	+
Ps. fluorescens	+	+	+	−	+	±	−	*−	−	*−	±	±
Pr. vulgaris	⊕	+	−	−	⊕	⊕	⊕	−	⊕	+	+	−
E. coli	⊕	+	⊕	*−	⊕	⊕	+	⊕	⊕	⊕	+	⊕

**: −, non acidity in 6 days; ±, weak acidity; +, acid produced; ⊕, acid and gas produced; *−, alkaline reaction.

and in all subsequent work are detailed on 15 strains which were selected as representatives of mineral requirement groups among all tested microorganisms were labelled as follows: 1007–1, 1040–1, 1055–1, 1055–2, 1064–2, 1135–4, 1179–2, and 1197–4 each strain from marine isolates; F–12 strain from the surface of squid; and *Vibrio parahaemolyticus* (0–5), *Vibrio metschnikovii* IAM-1039, *Pseudomonas fluorescens* FHU-*Ps.* 3, *Proteus vulgaris* NCTC-4175 and *Escherichia coli* (0–26) from named strains of terrestrial bacteria.

The localities of isolation and the results of morphological, cultural, and physiological tests of the selected strain are in Tables 3 to 6.

The tests might be inadequate to distinctly classify the isolates, but they will serve to practically define them. When the nine strains selected from marine isolates is classified according to the system outlined in Bergey's Manual of Determinative Bacteriology 7ed. (1957), they were tentatively identified as 1040–

1, 1055-1, and 1197-4 strain, *Pseudomonas*; 1007-1 and 1135-4 strain *Achromobacter*; 1055-2 strain, *Flavobacterium*; 1062-2 strain, *Vibrio*; 1179-2 strain, *Micrococcus*; and F-12 strain, *Photobacterium*.

II. Survey of mineral requirements of test microorganisms

Generally, the inorganic salt requirements of bacteria are not well understood. The chief obstacle in the work of this nature is the difficulty of obtaining a sufficient medium, free from inorganic contaminants to permit accurate observations.

In the previous paper (Hidaka, 1964), the author had been tried to design a common basal medium for various bacteria isolated from different origins, and established a basal medium for comparison of mineral requirements and salt tolerance of the microorganisms. It is a diluted nutrient medium containing 0.05 per cent of polypeptone and 0.01 per cent of yeast extract in pure water. Polypeptone was used to provide amino acids, small amount of sugar, and phosphates for the medium, and yeast extract was used to provide an array of vitamin and trace elements. Although most media for the growth of heterotrophic bacteria contain from 0.1 to 1.0 per cent of organic matter, it is generally claimed that the minimum concentration required for their multiplication ranges between 0.001 and 0.01 per cent (in ZoBell and Grant, 1942). As stated in the previous paper, the polypeptone and yeast extract included some inorganic contaminants. The contaminants were reluctuntly put in media prepared with the organic matter, and the accurate results were lost in special experiments concerning mineral requirements and salt tolerance. Therefor the author had been designed the diluted nutrient medium to reduce the inorganic salt contaminants from polypeptone and yeast extract by dilution of the organic matter concentration in the media. The test organisms grew satisfactorily in the diluted nutrient medium. Moreover, It was found that application of the medium may give more correct results than in the ordinary media in experiments concerning mineral requirements.

Here, the mineral requirement and salt tolerance were observed on the cultures grown in basal medium adding various inorganic salts and have various salt concentrations.

1. Growth responses of test microorganisms in five types of defined media

In this experiment, pure water, 0.5 per cent NaCl solution, 3 per cent NaCl solution, artificial sea water diluted six-fold, and artificial sea water were used for dilution of the diluted nutrtrient broth. The artificial sea water (A. S. W.) was prepared according to Herbst's formula with the following contituents (g per liter): NaCl, 30.0 (0.51 M); KCl, 0.7 (9.8 mM); $MgCl_2 \cdot 6H_2O$, 10.8 (0.052 M); $MgSO_4 \cdot 7H_2O$, 5.4 (0.021 M); $CaCl_2 \cdot 2H_2O$, 1.0 (7.3 mM), the final pH was adjusted to 8.0 (in Galtsoff *et al.*, 1937).

The author found the following correlations between supplementary roles of salts by the diluents, that pure water does not supply minerals. Both 0.5 and 3 per cent NaCl solutions supply only NaCl of its own concentrations, both one-six fold and full strength artificial sea water each supply concentrations of its own minerals, and 0.5 per cent NaCl solution make a medium similar to the salt content of ordinary broth. On the other hand, the osmotic action of 0.5 per cent NaCl solution and 3 per cent NaCl solution are isotonic to that of artificial sea water diluted six-fold and artificial sea water.

Experimental methods

The media employed in this experiment were composed of 0.05 per cent polypeptone and 0.01 per cent of yeast extract, and these ingredients were dissolved in the diluents of five types, and all media were adjusted at pH 7.8 with NH_4OH solution. In this case, the five types of defined media means the five media, and each medium of these were designated as (a), (b), (c), (d), and (e) medium.

Inocula. Media consisting of 0.5 per cent polypeptone and 0.1 per cent yeast extract were found to be suitable for the growth of test organisms. For the marine isolates, artificial sea water, for the terrestrial bacteria, 0.5 per cent NaCl solution, and for *V. parahaemolyticus*, 3 per cent NaCl solution were used as the diluent of culture medium. Inocula were made from cultures at 25°C for 24 to 48 hours on the above mentioned agar slants, and the cultures were transferred to each assay tube with a platinum wire giving an initial poputlaion of 10^3–10^4 cells per ml.

Assay procedure. The cultures inoculated in test media were incubated at 25°C for 6 days. Growth in test media was measured turbidimetrically in an AKA model No. 5, D colorimeter at 630 mμ. Some of the organisms incubated in the diluted nutrient media began to autolyse after completing their growth. Consequently, unless otherwise indicated, the author observed the growth exent of each bacterium every day during the entire incubation period, and their results were described with maximum turbidity. The culture tubes were pyrex test tubes (outer diameter, 18 mm) selected to fit into the colorimeter.

Experimental results

The results obtained are given in Table 7.

As shown in Table 7, terrestrial bacteria are able to grow in all types of defined media. In the case of terrestrial bacteria, to grow in the basal medium [(a) medium] means to require less minerals, and as a result, they are able to grow in nutrient broth without any supplement of inorganic salts, because in such medium, accountable inorganic salts needed for their growth are usually contained as contaminants of organic matter. And then most terrestrial bacteria also grew in salty media similar to sea water in their concentrations.

V. parahaemolyticus, *V. metshnikovii*, 1007–1, 1064–2, 1179–2, and F-12 strains each lack in the capacity to grow in the (a) or (a) and (b) medium. They seemed to require NaCl for their normal growth. The majority of marine isolates did not

Table 7. Growth extents of test organisms in five types of defined media
The growth extents (-Log T×100) indicate the maximum turbidity during 6 days incubation at 25°C.

Strain	Types of defined media*				
	(a)	(b)	(c)	(d)	(e)
1007-1	0	0	3	15	17
1040-1	0	0	0	15	45
1055-1	0	0	0	35	43
1055-2	0	0	0	9	24
1064-2	0	0	3	16	18
1135-4	13	13	15	20	21
1179-2	0	0	17	30	45
1197-4	0	0	0	30	38
F-12	0	0	3	1	5
V. parahaemolyticus	0	12	33	27	46
V. metschnikovii	0	10	9	12	14
Ps. aeruginosa	19	22	15	31	30
Ps. fluorescens	15	18	15	22	16
Pr. vulgaris	10	11	0	19	10
E. coli	14	14	12	22	15

*: All types of media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract and dissolve in following diluents: (a) pure water, (b) 0.5% NaCl solution, (c) 3% NaCl solution, (d) artificial sea water diluted six-fold, (e) artificial sea water.

The artificial sea water was prepared according to Herbst's formula, constituents are as follows (g per liter): NaCl, 30.0 (0.51 M); KCl, 0.7 (9.8 mM); $MgCl \cdot 6H_2O$, 10.8 (0.052 M); $MgSO_4 \cdot 7H_2O$, 5.4 (0.021 M); and $CaCl_2 \cdot 2H_2O$, 1.0 (7.3 mM), the final pH was 8.0.

grow in the medium (a), (b), and (c). They require not only NaCl but also K-, Mg-, and Ca-salt contained in sea water. It is ascertained that marine isolates have a strong requirement for many salts contained in sea water.

According to these results, growth effect of minerals on many bacteria appear markedly in diluted nutrient media, and such media are recommended as one of the useful media in this experiment.

2. *Influence of temperature and pH upon the growth of microorganisms in the five types of defined media*

In order to elucidate the effect of the temperature and pH upon the bacterial growth in the five types of defined media, each growth of test organisms in the defined media was observed at various temperatures or at differerent pH values.

The growth extents of test organisms incubated at different temperatures 6, 15, 20, 25, 30, and 37°C for 6 days were measured by the previously described procedure. The results obtained are shown in Table 8. In this experiment, the following results were obtained; that in both groups marine isolates and terres-

Table 8. Growth extents of test organisms in five types of defined media during 6 days incubation at various temperatures (Maximum tubidity: -Log T×100)

Types of defined media* / strain	Incubation temperature (°C)																													
	6					15					20					25					30					37				
	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)
1007-1	0	0	0	3	11	0	0	11	21	22	0	0	13	17	22	0	0	3	15	17	0	0	3	12	14	0	0	0	0	13
1040-1	0	0	0	3	40	0	0	0	35	54	0	0	0	34	45	0	0	0	15	45	0	0	0	0	40	0	0	0	0	0
1055-1	0	0	0	9	42	0	0	2	31	53	0	0	0	42	46	0	0	0	35	43	0	0	0	1	34	0	0	0	0	0
1055-2	0	0	0	0	3	0	0	0	17	28	0	0	0	22	23	0	0	0	9	24	0	0	0	0	24	0	0	0	0	0
1064-2	0	0	0	0	1	0	0	0	13	9	0	0	0	23	25	0	0	6	16	18	0	0	0	20	12	0	0	0	0	15
1135-4	1	0	0	1	1	4	10	0	13	19	7	12	0	5	25	13	13	15	20	21	10	8	7	15	18	13	5	0	11	9
1179-2	0	0	0	3	6	0	1	10	23	33	0	6	8	41	41	0	0	17	30	45	0	0	19	24	42	0	0	0	0	10
1197-4	0	0	0	5	27	0	0	0	34	48	0	0	0	43	39	0	0	0	30	38	0	0	0	0	38	0	0	0	0	0
F-12	0	0	0	1	1	0	0	0	2	0	0	0	0	3	2	0	0	3	1	5	0	0	0	0	3	0	0	0	0	0
V. parahaemolyticus	0	0	0	1	2	0	0	15	20	28	0	31	30	32	45	0	12	33	27	46	0	26	30	30	45	0	0	17	24	32
V. metschnikovii	0	0	0	0	0	0	16	22	15	23	0	10	9	12	14	0	10	9	12	14	0	15	14	22	19	0	0	4	5	6
Ps. aeruginosa	0	0	0	0	0	15	15	16	21	13	32	13	38	23	38	19	22	15	31	30	25	18	32	17	30	20	16	17	13	19
Ps. fluorescens	0	0	0	2	1	12	10	17	29	6	19	25	19	51	17	15	18	15	22	16	18	18	17	31	22	0	12	7	18	7
Pr. vulgaris	1	3	0	0	0	9	26	2	18	8	15	18	0	23	13	10	11	0	19	10	12	14	0	22	8	0	0	0	11	0
E. coli	0	0	0	0	0	12	15	4	20	18	21	17	13	22	15	14	14	12	22	15	15	19	10	20	14	10	10	7	12	9

*: See Table 7.

Table 9. Growth extents of test organisms in five types of defined media during 6 days incubation at 25°C in different pH values (Maximum turbidity: -Log T × 100)

Types of defined media* / Strain	pH value of the media																									
	6.0					6.5					7.0					7.5					8.0					
	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	(a)	(b)	(c)	(d)	(e)	
1007-1	0	0	0	4	9	0	0	0	9	14	0	0	0	11	11	0	0	5	10	11	0	0	8	10	14	
1040-1	0	0	0	16	33	0	0	0	17	34	0	0	0	18	32	0	0	0	21	33	0	0	0	21	31	
1055-1	0	0	0	0	31	0	0	0	34	36	0	0	0	31	39	0	0	0	32	37	0	0	0	30	36	
1055-2	0	0	0	0	28	0	0	0	12	28	0	0	0	13	28	0	0	0	14	27	0	0	0	11	27	
1064-2	0	0	0	14	19	0	0	0	14	18	0	0	0	14	17	0	0	0	13	16	0	0	0	17	17	
1135-4	1	4	7	11	20	1	4	5	11	18	3	4	5	13	18	1	3	6	13	14	1	3	6	12	17	
1179-2	0	0	0	0	0	0	0	0	0	23	0	0	0	16	36	0	0	3	20	34	0	0	17	30	39	
1197-4	0	0	0	22	30	0	0	0	23	28	0	0	0	28	29	0	0	0	22	30	0	0	0	28	25	
F-12	0	0	4	0	6	0	0	3	0	6	0	0	2	0	6	0	0	3	2	7	0	0	3	1	6	
V. parahaemolyticus	0	0	32	29	39	0	0	31	30	43	0	0	31	31	42	0	12	32	31	40	0	12	30	32	40	
V. metschnikovii	0	0	0	17	15	0	0	17	15	17	0	6	12	15	14	0	9	13	15	17	0	6	12	15	13	
Ps. aeruginosa	21	17	14	24	24	21	16	15	24	24	19	15	14	25	24	20	12	14	25	26	19	13	13	23	25	
Ps. fluorescens	19	17	15	30	11	19	19	16	32	13	22	18	11	30	15	18	21	18	29	17	21	18	10	30	18	
Pr. vulgaris	0	11	0	15	7	10	12	0	16	8	12	13	0	18	7	10	15	0	17	9	10	14	3	18	9	
E. coli	17	20	16	23	14	15	20	17	22	15	17	19	18	19	27	18	20	17	22	15	18	20	18	22	14	

*: See Table 7.

trial bacteria optimal temperature for maximal growth extent measured every day for 6 days, may be lower in temperature than those measured with generation times. Really, marine isolates grow well at temperatures from 15° to 25°C and the terrestrials from 20° to 30°C. It has been known that the salinity optima for marine fungi and some halophilic bacteria were affected by cultural temperature (Ritchie, 1957; Gibbons *et al.*, 1961). In this result, optimal temperature for growth extents of tested organisms changed in each type of the defined media. And growth extents of test bacteria in each of defined media were changed by incubation temperature. For example, 1055-1, 1055-2, and 1197-4 strains grew in both medium (d) and (e) at 15° to 25°C but did not grow in (d) medium at 30°C. Such a tendency was ascertained in most bacteria tested.

The effect of pH on growth extents is shown in Table 9. Adjustments in pH of media were made simply by adding NH_4OH or HCl. Ascertainment of pH was made with a glass electrode pH meter model HM-5A (Tōa Denpa Kōgyō Co., Ltd., Japan). In this cultural condition, most organisms tested, uniformly grew in the media in a wide range of pH from 6.0 to 8.0. Effects of pH on bacterial

growth was not detectable in either the marine isolates or the terrestrial bacteria.

The pH encountered in the sea ranges from 7.5 to 8.5. When the water is in equilibrium with the CO_2 of the atmosphere, the pH of sea water ranges from 8.1 to 8.3. In the euphotic zone photosynthetic plants may reduce the CO_2 content of the water until the pH reaches 8.3 to 8.5 during the hours of intense sunlight. Below the euphotic zone the pH generally decreases with depth to a minimum of 7.5 at depths exceeding 1,000 meters. Therefore, it is generally thought that in the sea water the pH cannot be a limiting factor for the development of microorganisms.

The growth extents of test bacteria in each of the five types of defined media were changed by pH values of the media. Many bacteria cultured in defined media (a), (b), (c), (d), and (e) have a special pH-sensitivity to their growth. They grow well in wide extent in the alkali side, while they grow in a narrow extent in the acid side.

As shown in the results of Tables 7, 8 and 9, tested bacteria are grouped into the following three types by means of their mineral requirement.

Bacteria of the first type are ones growing in all five defined media (a), (b), (c), (d), and (e). As they have only a few requirement for minerals, they are able to grow easily in the five defined media. Such types are the same as land types of bacteria. Bacteria of the second type grow in either of the defined media (b), (c), (d), or (e) except (a), or either (c), (d) or (e) except (a) and (b). Such types are the same as NaCl requiring bacteria. Bacteria of the last type grow in both of the defined media (d) and (e) excepting the other three. Such bacteria have specially require to minerals. Not only NaCl but also the major minerals in sea water are needed for their growth.

As previously mentioned in this chapter, if one trys to decide the mineral requirements of microorganisms using the defined media, he must keep the culture condition especially pH and temperatures correct. Bacause, in this experiment using the defined media, it is presumed that the questionable result is obtained by differences of culturing temperatures and adjusted pH. For the purpose of this experiment, the author proposed that recommended culture conditions are as follows: Incubation temperature is 25°C; adjusted pH is 7.8.

3. Salt tolerances of test microorganisms in the diluted nutrient medium

The ability of multiplication on test organisms was tested in different salt concentrations. In this test, NaCl was used in 0, 0.5, 1, 3, 5, 7, 10, 12, 15, and 20 per cent (W/V), and the other minerals (KCl, $MgCl_2$, $NgSO_4$, and $CaCl_2$) were also added to artificial sea water levels. The 3 per cent NaCl solution mixed with the other minerals equal to the composition of artificial sea water, and at the same time 0.5 per cent NaCl with the other minerals equal to the composition of artificial sea water diluted six-fold. Above 3 per cent NaCl solution with the other minerals increase only NaCl concentration and uniformly contained mixture

Table 10. Growth extents of test organisms in the diluted nutrient medium containing NaCl in different concentrations

Strain	Growth (Maximum turbidity: -Log T×100) at 25°C for 10 days														
	Per cent of NaCl in medium														
	NaCl only						NaCl with the other minerals in A.S.W.								
	0	0.5	1	3	5	7	0	0.5**	3*	5	7	10	12	15	20
1007-1	0	0	1	3	3	2	0	15	17	16	16	15	14	11	5
1040-1	0	0	0	0	0	0	0	15	35	45	46	36	12	6	0
1055-1	0	0	0	0	0	0	0	35	43	45	44	39	31	2	0
1055-2	0	0	0	0	0	0	0	9	24	22	18	17	5	0	0
1064-2	0	2	4	6	7	4	0	16	18	16	16	11	5	0	0
1135-4	12	13	14	16	3	0	12	20	21	21	20	17	12	6	0
1179-2	0	0	10	17	5	2	0	30	45	37	29	20	8	2	0
1197-4	0	0	0	0	0	0	0	30	38	43	47	45	31	3	0
F-12	0	0	0	3	2	0	0	1	5	5	4	0	0	0	0
V. parahaemolyticus	0	26	30	33	32	27	0	27	46	44	38	5	0	0	0
V. metschnikovii	0	10	10	9	0	0	0	12	14	10	6	0	0	0	0
Ps. aeruginosa	21	22	21	15	8	0	22	31	30	17	0	0	0	0	0
Ps. fluorescens	18	20	18	15	6	0	18	22	16	8	0	0	0	0	0
Pr. vulgaris	12	22	16	0	0	0	12	19	10	3	0	0	0	0	0
E. coli	17	20	20	16	8	1	17	22	15	8	0	0	0	0	0

All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.

* This is equal to the composition of artificial sea water.

** The artificial sea water diluted six-fold, accordingly NaCl concentration is 0.5%.

of the other minerals in artificial sea water levels. Inocula were prepared and used as described in the examination in Section 1. The cultures incubated at 25°C, were observed every day for 10 days for growth extent.

The data are shown in Table 10. It was apparent that a majority of marine isolates could not multiply in the medium containing only NaCl the sole source of salts, even 3 per cent or more concentration of NaCl was not available for such bacterial growths of marine isolates. In the media containing NaCl in different concentrations prepared with ordinary mineral constituted artificial sea water, it was found that many marine isolates multiply in the medium containing 15 per cent NaCl, but the majority could not grow in 20 per cent NaCl medium. For the exception of marine isolates and terrestrial bacteria could grow in media containing only NaCl for their mineral source. These organisms grew better in the medium containing the major minerals in sea water than in the medium containing NaCl only, even NaCl concentrations are similar in levels. In this case, the effect

of artificial sea water means the effect of other minerals except NaCl. Growth of most terrestrial bacteria was not affected by using artificial sea water, because these bacteria grew similarly in the same NaCl concentrations in the culture medium, whether artificial sea water is supplemented or not. *Pr. vulgaris* is an exceptional one, it could not grow in the medium containing 3 per cent NaCl only, but grew in the same concentration of NaCl supplementing the other minerals in artificial sea water. It is known that the other minerals (i. e. KCl, $MgCl_2$, $MgSO_4$, and $CaCl_2$) in artificial sea water alleviate the interference of NaCl in growth of microorganisms.

In the majority of marine isolates, the optimum salt concentrations for growth was found to be 3 to 7 per cent. Terrestrial bacteria, on the other hand, showed a moderate growth without any supplement of inorganic salts to the basal medium, and the growth was almost entirely suppressed in a salt concentration of 5 to 7 per cent, the optimum is in 0.5 per cent.

4. *Grouping of microorganisms on the basis of their growth capacity manifested in the five types of defined media*

In this chapter, the author examined growth responses of test organisms in the five types of defined media (Table 7), growth at 37°C (Table 8), salt tolerance, and optimum salt concentration for their growth (Table 10). In the experiments, the author found that results obtained have some correlation with one another. The relationships are briefly noted in Table 11.

The results above mentioned tell us that one is able to group the tested organisms into three patterns by growth extent in five defined media. The first is the most popular group to which most marine isolates belonged. The speciality of this group is explained by the organisms not grown in either (a), (b), or (c) medium. The speciality of the second group is explained by the organisms not grown in (a) medium or both (a) and (b). Bacteria grown in all five defined media belong to the last group, and also terrestrial bacteria are one of this type. Each of the three groups were designated as Marine (M-) type bacteria, Halophilic (H-) type bacteria, and Terrestrial (T-) type bacterria.

Most Marine type bacteria grew best in a concentration of NaCl and other minerals in the culture medium, the former is from 3 to 5 per cent and the latter is the same as in the artificial sea water levels. Some of them grew best at 5 to 7 per cent. The maximum salt concentration tolerated in any growth was 12 to 15 per cent. A very few of them grew at 37°C. Of 89 strains of Marine type bacteria, 7 strains were grown at 37°C.

Some of terrestrial and marine isolates are classified in the group, Halophilic type. The majority of the Halophilic type grew best in a 3 per cent salt concentration. Salt tolerances of Halophilic type varied by the origin of each strain. In general, the strains of marine origin tolerated any growth in 12 to 15 per cent of salt concentration, and the terrestrial ones tolerated their growth in 7 per cent.

Table 11. Relationship between each of the three groups of test organisms and some cultural characteristics

Strain	Growth at 25°C for 6 days (Maximum tubidity : -LogT×100) Types of defined media* (a)	(b)	(c)	(d)	(e)	Optimum salt conc. for growth (NaCl %)**	Salt tolerance** (NaCl %)	Growth at 37°C	Grouping
1040-1	0	0	0	15	45	3-5	15	−	Marine (M-) type
1055-1	0	0	0	35	43	5-7	15	−	
1055-2	0	0	0	9	24	3-5	12	−	
1197-4	0	0	0	30	38	3-5	15	±	
1007-1	0	0	3	15	17	3-5	20	+	Halophilic (H-) type
1064-2	0	0	3	16	18	3	12	+	
1179-2	0	0	17	30	45	3-5	15	+	
F-12	0	0	3	1	5	3	7	−	
V. parahaemolyticus	0	12	33	27	46	3	10	+	
V. metschnikovii	0	10	9	12	14	3	7	+	
1135-4	13	13	15	20	21	3-5	15	+	Terrestrial (T-) type
Ps. aeruginosa	19	22	15	31	30	0.5	5	+	
Ps. fluorescens	15	18	15	22	16	1.0	5	±	
E. coli	14	14	12	22	15	0.5	7	+	
Pr. vulgaris	10	11	0	19	10	0.5	5	+	

*: See Table 7. **: with the other salts in A.S.W. levels
♯: Maximum salt concentration (%) tolerated with any growth

Nearly all strains of terrestrial bacteria belonging to Halophilic type grew at 37°C, but about half the number of marine isolates of Halophilic type could not grow at 37°C. Terrestrial bacteria belonging to Terrestrial type grew best in 0.5 per cent of salt concentration, and marine isolates of the type grew at 1 to 3 or 3 to 5 per cent. The former tolerated their growth at 5 to 7 per cent, and the latter tolerated their growth in 12 to 15 per cent. Nearly all strains of this type could grow at 37°C, without distinction between terrestrial and marine isolates.

The surface temperatures of sea water vary with season and latitude. Tropical waters in the open sea may have surface temperatures as high as 28° to 30°C, while in polar sea water temperatures approximate the freezing point of the water. The temperature range of the marine environment, −2° to 30°C is a

small contrasted with the range of air temperatures, −65° to 65°C. The temperature range of water exceeding 1,000 meters in depth is 5° to −1.5°C. Thus, about 90 per cent of the marine environment is perpetually colder than 5°C (in ZoBell, 1946). And as shown by ZoBell and Conn (1940), most marine bacteria could not grow at temperatures as high as 30°C, and 37°C may be lethal.

According to Sverdrup *et al.* (1949), unless diluted by heavy rainfall, melting ice, or rivers, the salinity of surface sea water generally ranges from 33 to 37 per mill. The salinity of deep or bottom water of the oceans varies within narrow limits, approximately 34.6 to 35 per mill. Sea water is a physiologically balanced salt solution containing more than half of the known elements. It is a diluted solution of several salts with some dissolved gases and trace of a vast number of orgainc compounds. Except for a few constituents which are produced or consumed by biological agencies, the composition of sea water is relatively constant.

The chlorine and bromine in sea water occur almost exclusively as chloride and bromide anions. Similarly sodium, magnesium, calcium, potassium, and strontium occur as cations. The ratios of the three principal anions of sea water, i. e., carbonates, sulfates, and chlorides in sea water are the reverse of the ratios of these anions in river water:

	%Carbonate	%Sulfate	%Chloride
River water	80	13	7
Sea water	1	11	88

Similarly the ratios of cations particularly sodium and calcium, are different in sea water and in river water:

	%Calcium	%Sodium	%Magnesium	%Potassium
River water	57.7	26.8	9.5	6.0
Sea water	3.2	83.7	10.1	3.0

The ratios are calculated from data by Clarke (1924) on the analysis of river water and sea water (in ZoBell, 1946).

Therefore, the author takes care under due consideration on effect of the specific marine environment to the multiplication or growth of microorganisms, and he knows that most of the bacteria exclusive of adventitious contaminants found in the sea are specifically marine type bacteria. This conclusion is substantiated by the fact that commonly known species of terrestrial bacteria such as members of the coli-form, bacilli, or Gram-positive cocci groups have been found in the sea relatively near land, and a few been found in open oceans.

Although organisms belonging to these three groups, Marine, Halophilic, and Terrestrial type have been found widely in the sea, the author believes that only the Marine type bacteria in these three types which require not only NaCl but also K-, Mg-, and Ca-salt contained in sea water and can not grow at 37°C, should be designated "marine bacteria" in the strict sense.

III. Distributions of microorganisms belonging to Marine, Halophilic, and Terrestrial type

In the previous chapter, the author proposed that the microorganisms are grouped to the three by the behavior of mineral requirements estimated for their growth responses in the five types of defined media. The three types mentioned above are Marine, Halophilic, and Terrestrial type. All strains of terrestrial and marine isolates tested in this study were grouped by the procedure stated above.

In this chapter, the author tried to explain the relation between the origin or morphology and locality of isolation of test organisms of each type.

1. Distribution of microorganisms belonging to M-, H-, and T-type in different origins and morphologies

Of the test organisms in this study, 350 strains were grouped to M-, H-, and T-type by the procedure above mentioned. There were 275 strains of bacteria from sea water, 13 from the surface of fishes, 7 of NCMB strains, 12 of *V. parahaemolyticus* (0–1 to 0–12) and 6 of the similar, and 37 of named strains of terrestrial bacteria. Among the three types, T-type bacteria made a further distinction between the strains by dividing into two types, one type similar to *Pr. vulgaris* in Table 7, did not grow in the medium prepared with 3 per cent NaCl as a sole source of minerals, but the other T-type strains grew in the medium.

Many strains belonging to M-, H-, and T-type in different origins and morphologies are shown in Table 12. As seen in Table 12, 275 strains from sea water are distinguished into the following morphological parts: Gram-negative non-motile rods, 47; Gram-negative motile rods (polar flagella), 66; Gram-negative motile rods (peritrichous), 28; Gram-positive non-motile rods, 54; Gram-positive rods, 5: Gram-positive cocci, 75 each strain. Numbers of the distributed strains in M-, H-, and T-type were as follows: M-type, 89 strains (32 per cent); H-type, 48 (18); and T-type, 128 (50). Distribution of the three types M-, H-, and T-type were studied on morphologicaly different groups. It was found that the distribution rate of the M-type was much higher in the Gram-negative motile rods (polar flagella) than in the other groups. As compared with rods form bacteria, most of the cocci belonged to T-type, and about half of the number of cocci in the T-type have no-growth in the medium prepared with 3 per cent NaCl only, therefore, they have no tolerance to NaCl.

Many strains isolated from surface of fishes belonged to the T-type, since the fishes are caught off share.

Among 7 strains received from the National Collection of Marine Bacteria, 6 strains were Gram-negative polar flagella rods that belonged to the M-type. Another strain was Gram-negative peritrichous rods which belonged to the H-type.

Table 12. The number of strains belonging to M-. H-, and T-type of test organisms in different origins and morphology

Test organisms				Total no. of test strains	No. of strains belonging to the groups			
Origin	Morphology				M	H	T	
	Form	Gram stain	Flagella				Crowth at 3% NaCl	
							+	−
Sea water	Rods	−	−	47(100)	17(37)	11(23)	11(23)	8(17)
		−	+*	66(100)	47(71)	11(17)	7(11)	1(1)
		−	+**	28(100)	5(18)	12(43)	10(36)	1(3)
		Total		141(100)	69(49)	34(24)	28(20)	10(7)
		+	−	54(100)	12(22)	5(9)	30(56)	7(13)
		+	+	5	2		2	1
		Total		59(100)	14(24)	5(8)	32(54)	8(14)
		Sum total		200(100)	83(42)	39(19)	60(30)	18(9)
	Cocci	+	−	75(100)	6(8)	9(12)	20(27)	40(53)
	Total			275(100)	89(32)	48(18)	80(29)	58(21)
Surface of fishes	Rods	−	−	1	1			
		−	+*	6		3	3	
		−	+**	1				1
		+	−	2			1	1
		+	+	1			1	
	Cocci	+	−	2			2	
	Total			13	1	3	7	2
Sea	NCMB strains			7	6*	1**		
Feces or Surface of marine fishes	*Vibrio parahaemolyticus*			12		12		
	Similar			6		6		
	Total			18		18		
Soil, fresh-water, and enter	*Vibrio* spp.			2		2		
	Pseudomonas spp.			10		1	9	
	Achromobacter spp.			2			1	1
	Proteus spp.			7			5	2
	Escherichia spp.			2			2	
	Bethesda sp.			1			1	
	Bacillus spp.			7			6	1
	Micrococcus spp.			4			1	3
	Sarcina sp.			1				1
	Lactobacillus sp.			1				1
	Total			37(100)		3(8)	25(67)	9(25)

*: Polar **: Peritrichous

Figures in parentheses give percentage.

Table 13. Comparison of main morphological groups of bacteria in soil, lake water, and marine materials (From ZoBell, 1946)

Morphological group	Soil 1)	Soil 2)	Lake water 3)	Marine materials 4)
Gram-negative rods	36.1%	26.7%	95.5%	94.6%
Gram-positive rods	46.5	73.1	3.8	1.2
Gram-variable rods	9.4			0.9
Cocci	3.8		0.7	2.8
Others	4.2	0.2		0.5

1) 625 soil cultures examined by Taylor and Lochhead
2) 209 soil cultures examined by Topping
3) 671 cultures from fresh-water lakes examined by Taylor
4) 750 cultures from sea water and marine mud examined by ZoBell *et al.*

All of the *V. parahaemolyticus* and similar strains were H-type bacteria. In terrestrial bacteria tested three strains of the H-type were found in only genera of *Vibrio* and *Pseudomonas*, and most bacteria without exception belonging to the H-type were T-type bacteria. M-type were not found in terrestrial bacteria at all.

About 80 per cent of the marine species catalogued by ZoBell and Upham (1944) were Gram-negative rods. From the random examination of colonies which developed on plates as well as from the direct microscopic examination of marine materials stained by the method of Gram, it is estimated that approximatly 95 per cent of the bacteria occurring in the sea are Gram-negative. This compares favorably with the percentage of Gram-negative bacteria found in bodies of fresh water by Taylor (1942), and is more than twice as many as Taylor and Lochhead (1938) found in the soil (see Table 13). The majority of the bacteria found in the sea are actively motile. Flagella have been demonstrated on between 75 and 85 per cent of the pure cultures which have examined, and a somewhat higher percentage have been reported as motile. Spore-forming bacteria, which comprise an important part of the bacterial population in soil, are practically absent in sea water, although they may be present in considerable abundance in the sea bottom. Cocci and actinomycetes are of more limited occurrence in the sea than on the land (in ZoBell, 1946). It is well known that soil bacteria like bacillus and actinomyces maintain a superior position in their environment. In the same meaning, marine bacteria like some Pseudomonas and Vibrio have a priority in their life in the ocean environment. In other words, it may be supposed that there are specific microbial flora inhabiting the marine environment. As shown in Table 12, of 275 strains isolated from sea water, about 32 per cent are Marine type, about 50 per cent are Terrestrial type. In the ratio of separates, the T-type is much higher than the M-type. In observations of Gram-stain and flagellation, most bacteria of the M-type belonged to Gram-negative and flagellate ones.

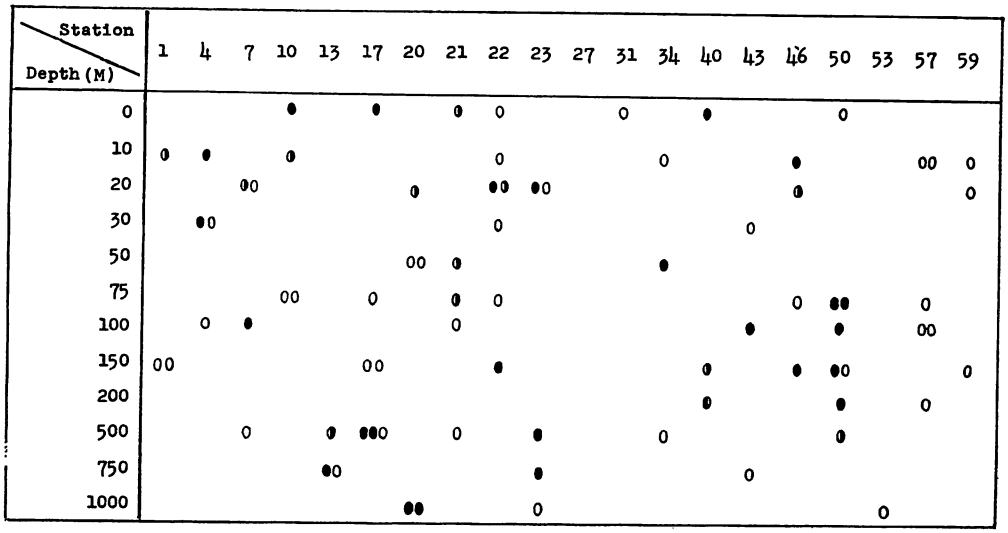
ZoBell and Upham (1944) and Kriss (1963) said in their descriptions that most bacteria collected from sea water are Gram-negative rods and the bacteria have monotrichous flagellum. As shown in the above mentioned experiment and the maniscripts of ZoBell *et al.* and Kriss, many types of bacteria, M-, H-, and T-type are known in sea water, but the most abundant type is the M-type. The M-type was not found in land or fresh-water habitats. This means, that the result is that a large number of bacteria from sea are carried considerably far inland by the wind, however they soon disappar into the environment of the more abundunt terrestrial ones. Except for the surface of marine fish or the media made by microbiologists, marine bacteria do not survive far inland beyond the shore line. On the other hand, it can be assumed that a number of T-type strains survive in sea water for long time, since there are many living cells of the T-type bacteria accounted in marine microbial flora.

2. *Vertical and horizontal distribution of marine isolates belonging to M-, H-, and T-type in the northern part of the North Pacific and the Bering Sea*

Near shore the movements of water, wind, migratory birds, and other agencies provide for a continuous interchange of microorganisms between the land and the sea. Since many fresh-water and soil bacteria can survive for prolonged periods of time in salt concentrations equal to or greater than that ocurreing in the sea, and since bays and estuaries provide a gradual transition from fresh to salt water, one might expect to find soil and fresh-water bacteria in the sea freely intermingled with marine forms.

Vertical and horizontal distribution of marine isolates belonging to M-, H-, and T-type in a region of the northern part of the North Pacific and Bering Sea are observed. The author has noted briefly in Figure 2 part 1 to 4 on the locality of marine isolates. In general, it is believed that a high percentage of M-type bactria are proved in the stations far from land. As shown in Figure 2, distances from land or islands and depth of sea seemed to have no clear relations with bacterial types. In open sea far from land some of T-type bacteria was detected.

A multiplicity of interrelated factors are known to be responsible for the number and variety of microorganisms found in an environment as complex as the sea. Therefore it is difficult to appraise quantitatively any one factor. The dynamic character of the marine environment further complicates the problem. Water masses are moving continuously. A water mass which is here today may be ten miles away tomorrow. In high latitudes, allowing for the movement of ice, pressing against the shore and subsequently driven out to sea by the variable winds, it could be admitted that soil bacteria could be transferred from dry land far into the sea. It can be assumed, therefore, that the microflora which occurred there was in the main composed of the survivors of the continental flora.



●: Marine type ◑: Halophilic type ○: Terrestrial type

Fig. 2. Locality of marine isolates belonging to the each of M-, H-, and T-types
Part 1. Gram-negative non-motile rods

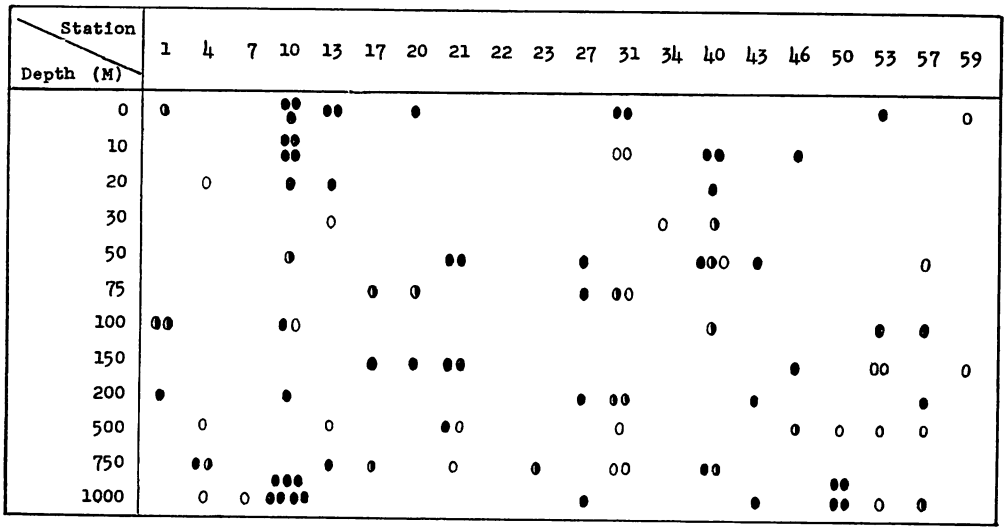


Fig. 2. (continued)
Part 2. Gram-negative motile rods

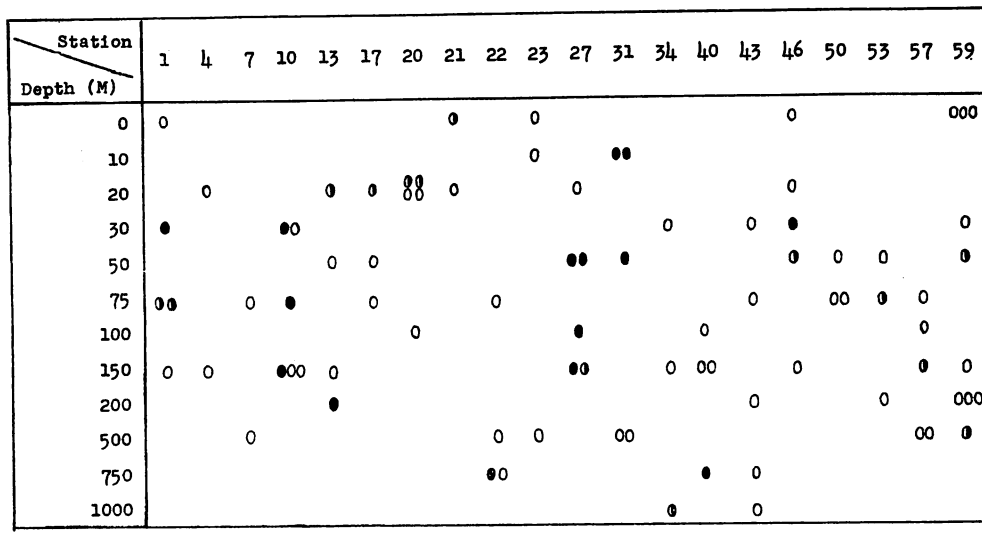


Fig. 2. (Continued)
Part 3. Gram-positive non-motile rods

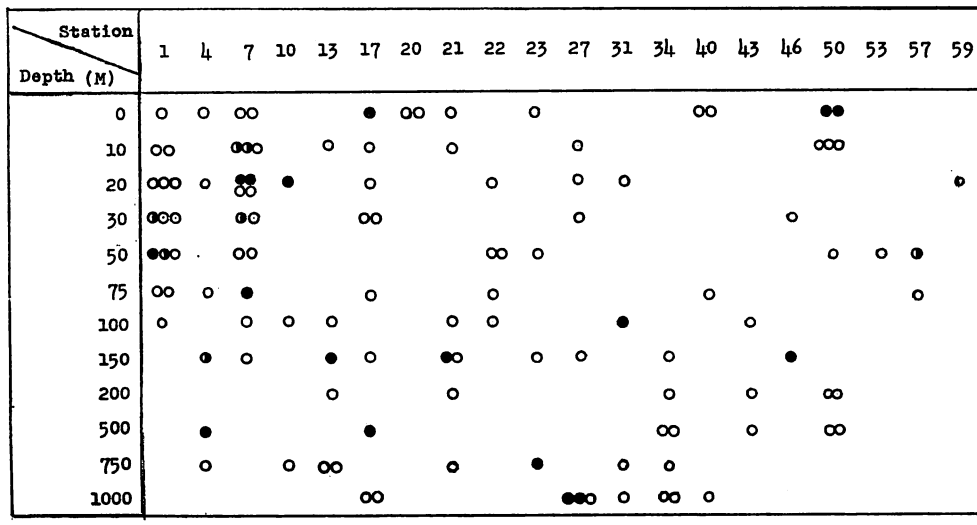


Fig. 2. (Continued)
Part 4. Gram-positive cocci

IV. On the specificity of mineral requirements of the Marine type bacteria

In the previous chapter, the author surveyed mineral requirements of tested microorganisms from various origins and grouped them into three forms by their growth response in the five types of defined media.

This chapter described on mineral requirement of Marine type bacteria especially on Sodium, Potassium, Magnesium and Calcium as major constituents of sea water. The author tried to explain the specificity of mineral requirement of tested organisms.

1. Influence of anions on the growth of test microorgasisms

MacLeod and Onofrey (1956) reported that, of six strains of marine bacteria tested by him, none of the organisms grew significantly without PO_4^{---} in the medium while the absence of SO_4^{--} prevented the growth of four organisms and reduced the amount of growth of the other two, and one organism required Cl^-, the growth of another was limited by its absence, while the remainder either were unaffected by its absence or needed Cl^- for it's optimum rate of growth. The author, therefore, observed the effect of Cl^- and SO_4^{--} on the growth of marine and terrestrial isolates in the basal medium used in this work.

Media and assay procedures

The author took precautions in the preparation of the basal medium, and the assay procedures used were the same as those applied previously, Chapter II. The basal medium contained 0.05 per cent polypeptone and 0.01 per cent yeast extract. Three media are as follows; one is made from basal medium plus artificial sea water, one is basal medium plus saline water replaced by corresponding chloride to sulphate in artificial sea water, and one is basal medium plus saline water replaced by corresponding sulphates to chlorides in artificial sea water. The test organisms were inoculated in the test media, and the cultures were incubated at 25°C for 6 days.

Experimental results

The results comparing the three media on growth promoting ability to tested organisms is shown in Table 14. As shown in Table 14, all the test organisms are able to uniformly grow in the media of the three types. The results obtained above mean that Cl^- and SO_4^{--} requirements of bacteria are the same as those due in the same anion that may be contained as contaminant in polypentone and yeast extract. There is no difference between terrestrial bacteria and marine isolates regarding the Cl^- and SO_4^{--} requirement.

2. Limiting concentration of inorganic salts on the growth of test microorganisms in the diluted nutrient medium

It has been reported that a number of marine bacteria require relatively high

Table 14. Effect of Cl^- and SO_4^{--} on the growth of test organisms

Strain	Growth (-Log T×100) at 25°C for 6 days		
	Artificial sea water*	Chlorides saline water**	Sulphates saline water***
1040-1	45	40	22
1055-1	43	31	15
1055-2	24	20	11
1197-4	38	35	25
1007-1	17	11	10
1064-2	18	12	12
1179-2	45	39	18
F-12	5	4	4
V. parahaemolyticus	46	55	35
V. metschnikovii	15	14	8
1135-4	21	14	10
Ps. aeruginosa	30	31	22
Ps. fluorescens	16	10	21
E. coli	15	13	11
Pr. vulgaris	10	3	6

All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.

*: Artificial sea water (A.S.W.), see Table 7.

**: The sulphates present in the A.S.W. were replaced by their corresponding chlorides.

***: The chlorides present in the A.S.W. were replaced by their corresponding sulphates.

Na^+ concentrations for their optimal growth and their metabolic activity. This requirement has been considered to be more than a simple expression of osmotic activity since the total replacement of Na^+ with nonspecific solutes has not been successful (Tyler *et al.*, 1960; Pratt *et al.*, 1963).

Here, the author tried to estimate the limiting concentration of inorganic salts on growth of test organisms and he observed whether the sea water is able to replace with glycerine, or not.

Assay procedures were similar to the above stated procedure.

First, test organisms inoculated in media prepared with suceessive diluted salts concentration of artificial sea water as diluents, and the cultures were incubated for 6 days at 25°C. If full strength artificial sea water used as the diluent in the medium, its concentration was considered to be 1 strength. Lower artificial sea water concentration, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 strength, were obtained by diluting the diluent appropriately with pure water. All media contained 0.05 per

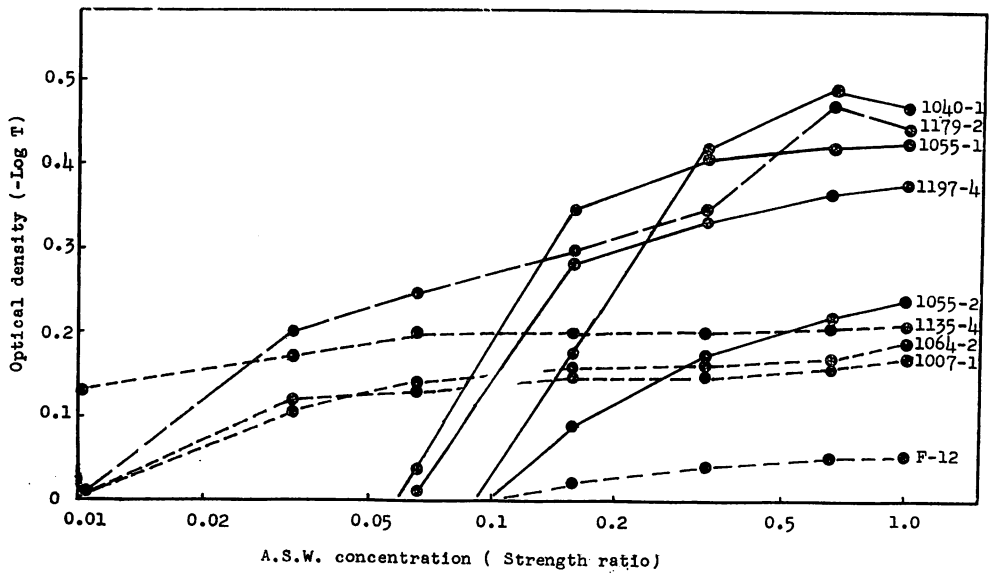


Fig. 3. Influence of the salt concentrations on the growth of marine isolates in artificial sea water (A. S. W.) media at 25°C for 6 days

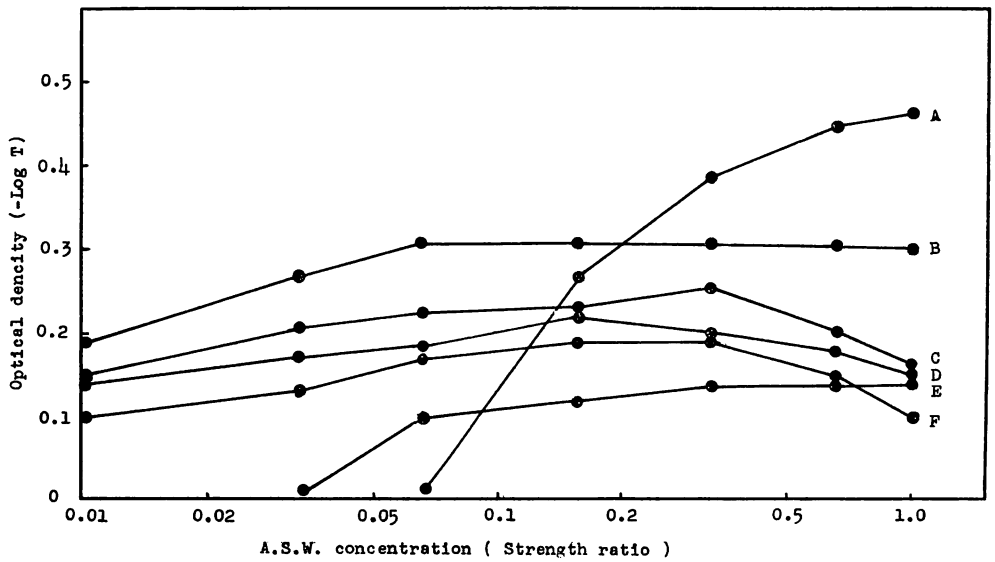


Fig. 4. Influence of the salt concentrations on the growth of terrestrial strains in artificial sea water (A. S. W.) media at 25°C for 6 days
Test strain: A, *V. parahaemolyticus*; B, *Ps. aeruginosa*; C, *Ps. fluorescens*; D, *E. coli*; E, *V. metschnikovii*; F, *Pr. vulgaris*.

cent of polypeptone and 0.01 per cent yeast extract, and the final pH was 7.8 to 8.0.

The effect of diluted salts concentration media on growth of tested organisms is shown in Figures 3 and 4. As shown in Figure 3, most marine isolates grew uniformly at range from 1 to 0.3 strength. M-type bacteria from marine isolates begin to decrease their growth extent in a salt concentration under 0.2 strength and finally they stop their growth in 0.1 to 0.05 strengths. On the other hand, in lower concentration such as 0.03 strength, H-type bactetia grew well enough, but their growth ceased in 0.01 strength. One exception, F-12 strain from H-type was not able to grow in 0.1 strength. It is assumed that the reason may be due to it's special qualities.

As shown in Figure 4, most named strains of terrestrial bacteria were able to grow enough even in 0 strength. A few exceptions were in the H-type bacteria named *V. metschnikovii* and *V. parahaemolyticus* belonging to terrestrial group. They cease their growth under 0.05 strength. It was known in this experiment that in diluted salt concentration in diluted nutrient artificial sea water broth, the M-type bacteria from marine isolates were not able to grow in 0.05 to 0.1 strength, and also the H-type bacteria were not able to grow in 0.01 strength, but the same type, H-type, bacteria from terrestrial group for example *V. metschnikovii* and *V. parahaemolyticus* were not able to grow in 0.05 strength. In the case of the M-type, possible limited concentrations for growth were 1/10 to 1/20 fold of artificial sea water, in the H-type it was 1/100 fold, and in the T-type supplements of artificial sea water were not needed for their growth.

Diluted mineral media made with various salt concentrations of each strength 0, 0.03, 0.06 and 0.15 were prepared by supplement of glycerine until the media became the same molar concentration as that in standard strength. Tested organisms were incubated in the media at 25°C for 6 days. On the other hand, the same experiment was carried out with the two different media described below for the sake of comparison. These were standard strength medium and diluted mineral media.

The results obtained are in Table 15. T-type bacteria not only grow well in 0-strength medium but also their growth was not affected by supplement of glycerine. Namely, they grew in all media used in this experiment. Of course, both M- and H-type bacteria from marine isolates did not grow in diluted mineral media, and the standard strength medium is only one of their growth media. Also they were not able to grow in isotonic media in which the osmotic pressure of the 0-strength medium was adjusted to that of the standard strength medium by glycerine. In the same isotonic media prepared with diluted mineral media and glycerine, M-type bacteria did not grow, as they did grow in the isotonic medium of 0-strength. It is an interesting problem that even same H-type bacteria of different origin whether land or marine shared different effects due to osmotic regulation.

Table 15. Growth responses of test organisms in the presence of glycerine in diluted salts media

Strain	Growth (Turbidity: -Log T×100)								
	Concentration of A.S.W. (Strength ratio)								
						(Glycerine added)**			
	1*	0	0.03	0.06	0.15	0	0.03	0.06	0.15
1040-1	45	0	0	0	18	0	0	0	10
1055-1	43	0	0	4	35	0	0	0	33
1055-2	24	0	0	0	9	0	0	0	13
1197-4	38	0	0	0	30	0	0	0	35
1007-1	17	0	12	13	15	0	8	10	13
1064-2	18	0	11	14	16	0	4	9	13
1179-2	45	0	20	25	30	0	6	18	28
F-12	5	0	0	0	1	0	0	0	7
V. parahaemolyticus	46	0	0	0	27	0	0	0	60
V. metschnikovii	14	0	0	10	12	0	0	6	10
1135-4	21	13	17	20	20	13	17	18	18
Ps. aeruginosa	30	19	27	31	31	22	45	42	40
Ps. fluorescens	16	15	21	22	22	21	39	35	28
E. coli	15	14	17	18	22	20	35	43	45
Pr. vulgaris	10	10	13	17	19	7	10	12	17

All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.

* Standard strength (artificial sea water)

** The osmotic pressures of the solutions were restored by adding a level of glycerine equal to the molar concentration of the standard strength.

Next, the media of different levels of other minerals except NaCl in artificial sea water were prepared for estimating the mineral requirment of microoranisms. Major minerals except NaCl are KCl, $CaCl_2$, and Mg-salts, and in this experiment NaCl was contained in equal quantities and only the other minerals were changed for the purpose of explaining the mineral requirements of test organisms.

Growth responses of test organisms in the media are shown in Figures 5 and 6. For the purpose of understanding the result of this experiment, mineral content of artificial sea water was standardized as 1, that of the media defined 0.5, 0.2… 0.001 strength.

As shown in Figures 5 and 6, most marine isolates grew regularly in the range from 1.0 to 0.05 strength media. The growth of the M-type bacteria decreased in the media under 0.02 strength, and growth ceased in 0.005 strength. Of course, the H-type bacteria were able to grow in 0 strength medium and the F-12 strain also grew in the same strength as the H-type in this experiment. Most terrestrial bacteria grew similarly in 1 to 0 strength media. One exception is *Pr. vulgaris* as described previously, could not grow in 3 per cent NaCl medium, but while in the same 3 per cent NaCl medium, adding to it other minerals i.e.

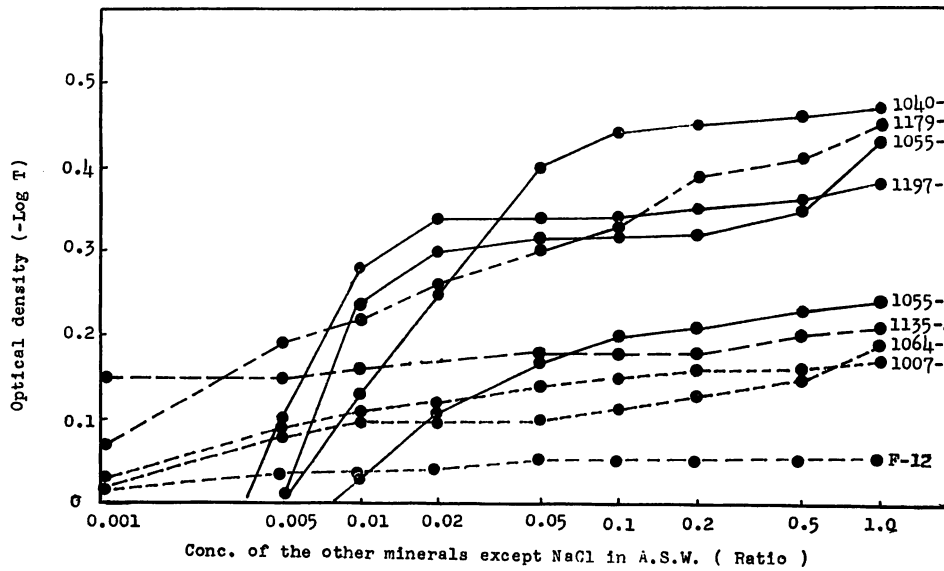


Fig. 5. Effect of the concentration of the other minerals except NaCl in artificial sea water (A. S. W.) on growth of marine isolates incubated at 25°C for 6 days

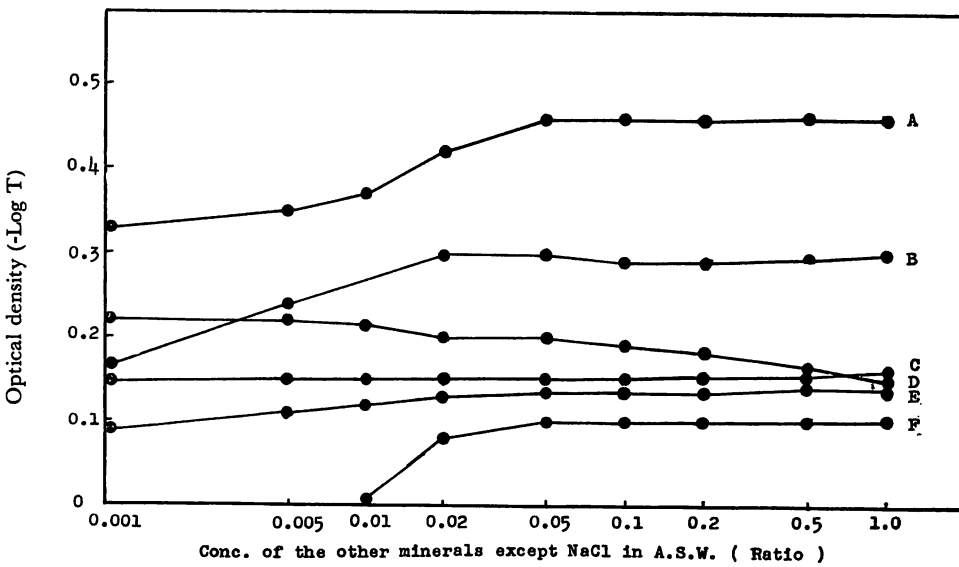


Fig. 6. Effect of the concentration of the other minerals except NaCl in artificial sea water (A. S. W.) on growth of terrestrial strains incubated at 25°C for 6 days
Test strain: A, *V. parahaemolyticus*; B, *Ps. aeruginosa*; C, *Ps. fluorescens*; D, *E. coli*; E, *V. mestchnikovii*; F, *Pr. vulgaris*.

KCl, $CaCl_2$, and Mg-salts, they grew appearantly. The bacterium also grew in all ranges from 1.0 to 0.02 strength, while they did not grow in 0.01 to 0 strength medium.

The results in Figure 5 and 6 show that the other minerals except NaCl were not indespensable for either bacteria, H- or T-types, while the M-type bacteria grew in 3 per cent NaCl medium containing from 0.01 to 1.0 strength the other minerals in artificial sea water. The M-type ones grew easily in the NaCl medium containing a small amount of the other minerals except NaCl in the artificial sea water. Therefore, it was ascertained that the M-type bacteria were the ones which required NaCl and the other minerals of artificial sea water as essential nutrients, and that limited concentration of growth is comparatively low.

The mineral requirement of the T-type bacteria was comparatively low. Even though without a supplement of inorganic salts in the basal medium, they were able to grow. To study more details of mineral requirements of T-type bacteria, the following experiment was planned. That is, in this experiment the limiting concentrations of Ca^{++} and Mg^{++} that are indespensable to growth were studied. To know the Ca^{++} and Mg^{++} requirements of bacteria, a chelate-reagent such as EDTA (Ethylenediaminetetraacetic acid) was added into the following medium, and the T-type bacteria were incubated in the medium. The preparation of the medium was as follows, polypeptone 0.5 g, yeast extract 0.1 g, and NaCl 5 g were dissolved in one liter of pure water, and the solution was adjusted to pH 7.8 to 8.0. Just before the examination 0.01 M EDTA was added as aseptically into the medium. In the medium bivalent cations such as Mg^{++} and Ca^{++} might be chelated with the EDTA, and so bacterial growth in the medium means the mineral requirement of the test bacteria in deficient minerals.

The results of the examination are shown in Table 16. In this experiment, when 2.0 ml of 0.01 M EDTA was used in the medium of 1 liter, the amount of

Table 16. Growth responses of some selected strains in the media chelated with inorganic contaminants in the basal medium by EDTA Growth (Maximum turbidity: -Log T × 100) at 25°C for 6 days

Strain	Volume (ml) of EDTA soln. (0.01M) added in the basal medium 1 liter						
	0	0.4	0.8	1.2	1.6	2.0*	2.4
1007-1	0	0	0	0	0	0	0
1135-4	13	13	5	4	0	0	0
V. parahaemolyticus	22	18	13	9	0	0	0
V. metschnikovii	10	10	10	6	6	4	0
Ps. aeruginosa	22	20	20	20	20	20	18
Ps. fluorescens	18	18	18	17	13	11	6
Pr. vulgaris	11	10	10	7	5	3	3
E. coli	14	14	14	13	12	11	11

*: Amount of EDTA is equivalent to the inorganic contaminants (Mg- and Ca-salt) in one liter of the basal medium.

EDTA was proportional to that of Ca^{++} and Mg^{++} in the medium. However, chelate condition between EDTA and each cation Mg^{++} or Ca^{++} is affected with pH, and the best conditions are known to be at pH 10.0 or 12.0. Under this experiment pH reached from 7.8 to 8.0, therefore it was impossible to know whether the best conditions were obtained or not under such experimental conditions. In spite of one's expectation that the pH of the medium was susceptible to decrease in the preparations, it was ascertained that the pH decrease in the medium was not proved in this experiment. All of the T-type bacteria grew in the culture media containing various ranges of EDTA. 1135–4 strain from marine origin belonging to the T-type grew in the medium containing EDTA of 1.2 ml/liter, but their growth ceased in the medium containing EDTA of more than 1.2 ml/liter. From the results of the experiments it was ascertained that the degree of mineral requirement of the same type of bacteria was not always equal to that of the others from their different origins.

3. *Bacterial growth in the media prepared with various combinations of mineral salts contained in artificial sea water*

Studies of mineral nutrition and osmotic relations have been reported for several marine bacteria. MacLeod and Onofrey (1954, 1956, 1957) revealed that none of the organisms grew unless Na^{+} and K^{+} were added to the medium, and additions of Mg^{++} were required by four of the organisms and were stimulatory for the growth of the other two, and Ca^{++} was required by one organism and stimulated the early growth of another. Only recently has intensive investigation been initiated with marine bacteria concerning those properties which distinguish physiologically from terrestrial bacteria. Tyler (1960) suggests that physiological versatility is a more common feature of marine isolates whose mineral nutrition can be satisfied by adding only Na^{+}, or $Na^{+}+K^{+}$ than of those requiring Mg^{++} as well.

The investigations reported here deal with the effects of major sea water minerals on several hundred strains of marine and terrestrial bacteria.

Experimental methods

Inocula and assay procedures were carried on in a similar method as previously described, Chapter II. The basal medium was a diluted nutrient medium containing 0.05 per cent polypeptone and 0.01 per cent yeast extract.

All solutions were prepared using water demineralized by passage through a monobed of ion exchange resins. In all cases, the inorganic salts used were of guaranteed reagent grade without further purification.

Results of experiment

Growth responses of test organisms obtained in diluted nutrient media containing the various combination of salts in the artificial sea water are shown in Table 17. The culture media were prepared to be the same molar concentrations as that of the original artificial sea water. That is one or more salts of the ar-

Table 17. Growth responses of test organisms to diluted nutrient medium containing various combination of salts in the artificial sea water

Media		A	B	C	D	E	F	G	H	I	J	K	L
Composition	NaCl 0.5132M	○	×	○*	○*	○*	○*	○*	○*	○*	×	×	×
	KCl 0.0098M	○	○*	×	○	○	○	×	×	×	○*	○*	×
	$MgCl_2$ 0.0525M	○	○	○	×	○	×	○	×	×	○	×	○*
	$MgSO_4$ 0.02159M	○	○	○	×	○	×	○	×	×	○	×	○
	$CaCl_2$ 0.00734M	○	○	○	○	×	×	×	○	×	×	○	○
Strain		Growth (Maximum turbidity: -Log T × 100) at 25°C for 10 days											
1040-1		45	0	40	42	40	21	32	8	0	0	0	0
1055-1		43	0	44	41	40	25	40	31	0	0	0	0
1055-2		24	0	21	22	15	0	22	24	0	0	0	0
1197-4		38	0	31	37	36	16	36	37	0	0	0	0
1007-1		17	0	19	17	15	5	14	12	3	0	0	0
1064-2		18	0	14	8	12	9	10	8	3	0	0	0
1179-2		45	39	29	39	42	28	26	26	17	43	41	13
F-12		5	3	5	7	6	6	3	4	3	0	0	0
V. parahaemolyticus		46	11	43	52	43	46	40	38	33	8	14	0
V. metschnikovii		14	2	10	15	13	8	10	10	9	0	8	0
1135-4		21	24	20	15	14	15	15	16	15	22	27	3
Ps. aeruginosa		30	31	38	34	35	34	36	21	15	7	8	15
Ps. fluorescens		16	15	12	14	12	14	13	15	15	5	3	3
E. coli		15	15	19	26	16	23	17	14	12	6	4	4
Pr. vulgaris		10	4	0	5	7	5	3	9	0	4	5	2

All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.
Composition — ○: added, ×: omitted, *: The molar concentration of this salt added in each case is equal to the total molar concentration of the salts omitted

tificial sea water were replaced with one of the other residual salts to reach the original molar concentration of artificial sea water.

As shown in Table 17, Terrestrial type strains grew well in all media except for one or two strains. This fact shows that many terrestrial bacteria did not essentialy require the mineral salts for their growth. And that both strains of the H-type, 1007-1 and 1064-2 strain, required NaCl and also other mineral salts could not replace their growth by NaCl. Other H-type bacteria each 1179-2, F-12, *V. parahaemolyticus*, and *V. metschnikovii* required some mineral salts, but their requirement was not so strict that the bacteria could not grow on the other mineral salts. In general, they were able to replace the minerals with other minerals for their growth. It was assumed that such requirement of mineral was due to non-specific function of the bacteria. The result did not agree with the previous data (see Table 15). That is, as shown in Table 15, the bacteria did not grow in an isotonic medium (osmotic pressure is equal to that of the artificial sea water medium) adjusted with glycerine, but grew well in another isotonic medium made

by other mineral salts. Eventually the H-type bacteria were divided into two groups, one was which required NaCl essentially and another one which required other mineral salts by non specific function.

As Marine type strains, it is evident that removing NaCl completely prevented the bacterial growth in all cases. Since NaCl constituent is approximately 80 per cent of all salts in sea water, one might expect that the effect of NaCl would be primarily due to the osmotic. NaCl, however, could not be replaced by other salts prepared in equimolar concentrations by one and more salts, and still more Marine type strains could not grow in the medium containing only NaCl as the sole source of mineral salt. NaCl and either Mg-, K-, or Ca-salt, or sometimes both or the mixtures of all salts were needed for promoting the Marine type strains.

For comparison of accelerative activity to NaCl by three salts, K-, Mg-, and Ca-salt, 10 days incubation was carried out and the result was shown in Table 17. In this table, not to say certainly, difference of the accelerative activity was comparatively distingushable in an initial growth phase obtained in 24 to 48 hours incubation. It was assumed in this experiment that in comparison of the accelerative activity of Mg-salt had the same high activity as Ca-salt, but K-salt was not equal to the former two in activity. Above mentioned accelerative effects of mineral salts on bacterial growth was different between the strains of bacteria. In the case of the Marine type, the growth of about 80 to 85 per cent strains with

Table 18. Growth responses of test organisms in diluted nutrient medium containing each single salt in artificial sea water

strain	Growth at 25°C for 10 days (Maximum turbidity: -Log T×100)			
	Added in 0.2 M to medium			
	NaCl	KCl	$MgCl_2$	$CaCl_2$
1040-1	0	0	0	0
1055-1	0	0	0	0
1055-2	0	0	0	0
1197-4	0	0	0	0
1007-1	0	0	0	0
1064-2	2	0	0	0
1179-2	6	5	7	0
F-12	2	0	0	0
V. parahaemolyticus	27	9	5	0
V. metschnikovii	10	10	1	0
1135-4	10	19	16	2
Ps. aeruginosa	20	2	5	0
Ps. fluorescens	18	3	7	0
E. coli	18	7	4	0
Pr. vulgaris	13	10	2	0

All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.

Mg-salt, that of about 70 per cent strains with $CaCl_2$ and that of about 70 per cent strains with KCl were accelerated. In strains numbers accelerated by the salts no difference was detectable, but in comparisons of growth effects by the three mineral salts KCl, $CaCl_2$, and Mg-salts, the first one was inferior to the last two.

Furthermore, the author observed the growth effects of single minerals and the result obtained was in Table 18. In this experiment, the molar concentration of the salts used were equivalent to one-third of that of artificial sea water. The salt concentrations of artificial sea water seemed to be too high for terrestrial bacteria and some questions was unavoidable for the study of the mineral requirements. As shown in Table 18, all of the M-type bacteria were not able to grow in a single salt medium.

NaCl was indespensable for growth of the M-type bacteria as shown in Table 17. The role of NaCl concerning the M-type bacteria was due not only to their non-specific osmotic function, but was also due to their true requirement of NaCl. To get details on NaCl requirements of the M-type bacteria, the author studied the effect of ions Na^+ and Cl^-. In this experiment, the next culture medium was prepared, that is, three media for checking the cations were prepared with the

Table 19. Growth responses of test organisms in the diluted nutrient medium containing some sodium salts and chlorides

Strain	Growth (Maximum turbidity: -Log T×100) at 25°C for 6 days									
	Single*					Single salt plus other salts**				
	NaCl	$NaNO_3$	Na_2SO_4	KCl	LiCl	NaCl	$NaNO_3$	Na_2SO_4	KCl	LiCl
1040-1	0	0	0	0	0	33	37	39	0	0
1055-1	0	0	0	0	0	41	51	42	0	0
1055-2	0	0	0	0	0	21	22	18	0	0
1197-4	0	0	0	0	0	37	41	36	0	0
1007-1	0	0	0	0	0	11	10	10	0	0
1064-2	3	0	0	0	0	16	16	18	0	0
1179-2	6	0	0	0	2	23	23	8	20	33
F-12	2	0	0	0	0	6	5	5	2	3
V. parahaemolyticus	27	18	29	3	0	43	30	38	16	25
V. metschnikovii	9	8	10	6	2	15	13	14	4	8
1135-4	10	10	7	16	6	16	19	21	18	10
Ps. aeruginosa	17	11	19	17	12	23	14	25	25	20
Ps. fluorescens	16	13	12	17	17	26	24	22	25	28
E. coli	18	16	18	22	15	18	14	19	18	20
Pr. vulgaris	13	12	16	17	19	18	14	17	16	18

All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.

*: The molar concentration of cation added in the medium is equal to 0.17 M. (nearly equal to one-third strength of molar concentration of total salts in sea water)

**: Single mineral plus the mixture of the other minerals except NaCl in artificial sea water diluted three times.

three Na-salts, each NaCl, $NaNO_3$ and Na_2SO_4, and on the other hand the three media for the anion were prepared with the three chlorides NaCl, KCl and LiCl. The other mineral salts except NaCl in artificial sea water were contained in the test media. Test organisms were incubated at 25°C for 6 days and the growth rate of the bacteria was measured.

The results are shown in Table 19. In this experiment, terrestrial bacteria were grown in every media and both *V. metschnikovii* and *V. parahaemolyticus* also grew in every media. Both strains seemed to belong to the T-type bacteria. Both the 1179–2 and the F-12 strains among the H-type bacteria not only grew in the NaCl medium, but also in the KCl and the LiCl medium. And both 1007–1 and 1064–2 strains seemed to be M-type bacteria in their growth appearance. M-type bacteria could not grow in only simple salts in any cases, but they grew easily in all of the Na-salts by a supplement of a mixture of the other salts. And also, the bacteria of the M-type were not able to grow in any single salt of KCl or LiCl even by a supplement of other salts without NaCl. Therefore, it was ascertained from this experiment that M-type bacteria took specical requirement of Na^+.

The results in Tables 17, 18 and 19 were summerized into the following that T-type bacteria are ones who do not take special requirements to mineral salts, and H-type bacteria are ones who require NaCl, but do not require any other minerals. The H-type bacteria were able to be divided into two groups, one was represented by both 1007–1 and 1064–2 strains and the other one by the strains each 1179–2, F-12, *V. parahaemolyticus* and *V. metschnikovii*. The former required NaCl indespensably and the NaCl requirement of the bacteria could not be replaced with any other mineral salts. The latter required NaCl but the NaCl requirement of the bacteria could be partialy replaced with other mineral salts, for example, the bacteria of the later grew partially in the medium of KCl or LiCl without NaCl, and it was then assumed that in this culture NaCl was replaced with KCl or LiCl. In other words, the minerals have some functions to their growth like osmolar control. Differences of mineral requirements between the group of 1179–2 or F-12 and another group of *V. parahaemolyticus* or *V. metschnikovii* were due to the distribution rate of their own requirements as like non-specfic and specific functions. That is, for the latter group the distribution rate of the non-specific function was superior to that of the specific function. The M-type bacteria only grew in NaCl and a few of the other mineral salts. In the case of M-type bacteria, Na^+ was indispensable to growth and it could not be replaced with any other cations. They also grew in $NaNO_3$ or Na_2SO_4 medium without NaCl, but only with the existence of K-, Mg-and Ca-salt. Again, in the case of the M-type bacteria Na^+ was essential for their growth, in the other words, they specially required Na^+ and the other salts in sea water in comparison with other bacteria which did not.

4. Effects of the salts contained in agar media on the bacterial growth

Plating procedures are one of the principal methods employed for estimating

the number of most bacteria in sea water. ZoBell and Upham (1944) defined marine bacteria as bacteria from the sea which on initial isolation require a medium containing sea water as the diluent. A large number of workers had used this definition for detection of marine bacteria. Tyler *et al.* (1960) had isolated marine bacteria from sea water by the following procedure. That is, the sample water was spread on the surface of sea water trypticase agar plates prepared with 1 per cent (W/V) trypticase and 2 per cent (W/V) agar in sea water. When some colonies developed on the plate after incubation at 27°C for 24 to 36 hours, replica plates were made on sea water-trypticase agar and on distilled water trypticase agar. Isolates were selected from the replica plates after incubation, with a choice being made from sea water plates of colonies which had no replicas on the distilled water agar medium.

In the present investigation, the author has found that a quantity of inorganic elements in a medium affect to both the rate and extent of growth of marine isolates. Since a medium was solidified with agar, the medium was easily contaminated by inorganic ions, because the chief contamination was due to the minerals contained in agar and other organic materials. By using the agar media without any considerations, therefore, accurate results could not be expected on the special examinations concerning mineral requirement and salt tolerance.

The author studied on the growth of bacteria on agar media using various salt solutions.

Methods

Media. The four media used were as follows; 0.5 per cent polypeptone, 0.1 per cent yeast extract and 1.5 per cent agar were dissolved in each of the following four solutes, pure water, 0.5 per cent NaCl solutioon, artificial sea water and 7 per cent NaCl artificial sea water. Finally each medium was adjusted at pH 7.6–7.8.

Observation. The streak or pouling culture incubated at 25°C, were observed for 6 days, relative growth was recorded according to visual judgment or counting of colony numbers.

Results

The development of marine isolates and terrestrial bacteria streaked on three types of agar media prepared with pure water, artificial sea water, and 7 per cent NaCl artificial sea water is shown in Table 20.

As shown in Table 20, T-type strains grew well in the pure water medium but did not grow in the 7 per cent NaCl artificial sea water medium. They grew faintly in artificial sea water agar. The H-type strains did not grow in the pure water medium but grew uniformly in both the 7 per cent NaCl artificial sea water medium and the artificial sea water medium. Also the M-type strains did not grow in the pure water medium but grew well in the artificial sea water medium and 7 per cent NaCl artificial sea water medium. The procedure was similar to Tyler's method above. According to the definition of ZoBell and Upham (1944)

Table 20. Growth of marine and terrestrial bacteria streaked on several types of agar media, at 25°C for 7 days

Types of agar media / Strain	5 g of polypeptone, 1 g of yeast extract, and 15 g of agar per liter		
	Pure water	A.S.W.	A.S.W. plus NaCl to 7%
1040-1	−	++++	++++
1055-1	−	++++	++++
1055-2	−	++	++
1197-4	−	++++	++++
1007-1	−	++	++
1064-2	−	++	+
1179-2	±	++	++
F-12	−	+	−
V. parahaemolyticus	−	++	++
V. metschnikovii	−	+	−
1135-4	++	++	++
Ps. aeruginosa	++++	+	−
Ps. fluorescens	++++	+	−
E. coli	++++	+	±
Pr. vulgaris	++++	+	−

Signs (−, ±, +, ++, ++++) represent degree of growth.

and the isolation method by Tyler *et al.* (1960) on marine bacteria, M-and H-types bacteria grouped by the author were included in the marine bacteria.

Next, the author carried out the experiment using the four agar media mentioned above. This experiment was conducted considering the direct separation of bacteria from sea water. That is, inoculum of each living cell was suspended in a concentration of 10^3–10^4 cells per ml in sterile artificial sea water, and at first one ml of the suspension was poured into an ordinary petri dish, next 15 ml of agar medium was added into the same petri dish. This experiment was carried out with four different agar media. The agar plate was immediatly incubated at 25°C for 6 days. After incubation colony numbers were counted on the plate, and within all plates each made from the different four media, the plate with the most abundant colonies was standardized. The numbers counted on the plate were regarded as 100 for comparison with other plates. The results obtained are in Table 21. The difference in experiments between Tables 20 and 21 seemed to be due to plate techniques, the former was by the streak culture technique and latter was by the agar pour plate technique. The streak culture technique does not give changes of medium concentration, but the agar pour technique gives changes, because the former technique is by streak on the agar surface, but the latter is diluted with the sample water. In the latter experiment, an agar medium of 15 ml and a sample of sea water of 1 ml were mixed, therefore, the concentration of the culture medium may be diluted with 1 ml of sample sea water. On the other hand, as the sample is of sea water, the mineral salts must be sup-

Table 21. Relative number (per cent) of colonies of marine and terrestrial strains which developed on nutrient agar prepared with various salt solutions

Types of diluents for agar media / strain	Pure water	0.5% NaCl solution	A.S.W.	A.S.W. plus NaCl to 7%
1040-1	0	0	100	100
1055-1	0	1	100	100
1055-2	0	0	100	90
1197-4	0	2	100	100
1007-1	100	100	100	100
1064-2	40	100	100	20
1179-2	80	100	100	60
F-12	0	10	100	30
V. parahaemolyticus	0	60	100	30
V. metschnikovii	80	100	70	0
1135-4	100	100	100	100
Ps. aeruginosa	100	100	100	0
Ps. fluorescens	100	100	50	0
E. coli	100	100	100	25
Pr. vulgaris	60	100	40	0

All media (pH 7.8) contain 0.5% of polypeptone, 0.1% of yeast extract and 1.5% of agar.

plemented into the medium.

In comparison with the data in Tables 20 and 21, apparently the differences were ascertained. That is in Table 20, M-and H-type strains seemed to be marine bacteria, while in the result in Table 21, M-type strains were unable to grow on either media, the pure water medium and 0.5 per cent NaCl medium and yet they grew similarly on both, the artificial sea water medium and the 7 per cent NaCl artificial sea water medium. On the other hand, the H-type strains were divided into two groups, one group grew well on all of four media and another group did not grow on the pure water medium but grew on the other three media. That is, by the agar pour plate technique, one can distinguish the M-type strains from the other H- and T-type bacteria. Therefore the bacterial populations of true marine flora and continental flora in sea water may be able to enumerate individually by this method.

V. Physiological meanings of specific mineral requirements for growth of the Marine type bacteria

A large number of reports have appeared concering the effect of salt concentra-

tion on the reaction of halotolerant and moderately halophilic bacteria (Flannery *et al.*, 1953; Flannery, 1956; Gibbons, 1957). Garrard and Lochhead (1939) found that many organisms, capable of reducing nitrate to nitrite in media containing 5 per cent salt, lost their reducing ability as the salt concentration was increased before they lost their ability to grow. Robinson (1952) showed that there was an optimum concentration of NaCl for maximum activity of lactic acid dehydrogenase. Dumesh noted decreasing the salt concentration of the the medium from 7 to 5 per cent was sufficient to stop the fermentation of glucose and lactose by some strains of colorless halophiles (in Gibbons, 1957). Only recently has intensive investigation been initiated with halophilic bacteria concerning the effect of various salts and salt concentrations on the morphology of the cells and cell walls, on the chemical composition of whole cells and cell walls, and the osmotic properties of the cells (Abram *et al.*, 1961; Takahashi, 1959). Many investigations were already known on halophilic bacteria, however, that of marine isolates was not known until comparatively late.

The present examinations were made on the effect of various salts and salt concentration on the physiological activity and osmotic fragility of selected marine isolates.

1. Effect of various kinds and concentrations of salt on biochemical activity of the strains selected from M-, H-, and T-type

In preliminary experiments, the usual medium for biological reactions was used by supplementing the various concentrations of mineral salts. In this method it was impossible to know the real mineral effect on biological activity, because of the irregurality of bacterial growth by mineral salt concentrations. For the purpose of removing the difficulty, the author used the intact cells in this experiment.

Methods of experiment

Concentrated intact cell suspensions 0.01 ml was added into one ml of defined substrated, dissolving the various mineral salts, and then, the reaction tubes were incubated at 30°C for 4 to 6 hours. After incubation the activities that appeared were measured by the usual method. Among the experiments, cytochrome oxidase activiety was measured by the reaction of 0.5 ml reagent. Constitution of the reagent was as follows; 2 parts of 1 per cent α-naphthol in 95 per cent alcohol solution and 3 parts of 1 per cent dimethyl-*p*-phenylene diamine-2HCl in a water mixed solution. Final cells concentration in test substrate was adjusted to 10^8 cells per ml.

In this chapter, the four tests, cytochrome oxidase, glucose decomposition, indole production, and nitrate reduction were examined. Used solutes for comparing the effect of mineral salts were of the following five, pure water, 0.5 per cent NaCl solution, 3 per cent NaCl solution, and artificial sea water diluted six-fold.

Results

Table 22. Effect of salts on glucose hydrolysis, cytochrome oxidase, production of indole, and reduction of nitrate by selected organisms

Test	Glucose hydrolysis					Cytochrome oxidase					Production of indole					Reduction of nitrate				
Saline water*	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
1040-1	−	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
1055-1	−	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
1055-2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1007-1	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	+	+	++	+	++
F-12	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	++	+	++
V. parahaemolyticus	−	+	+	+	+	−	+	+	+	+	−	+	+++	+	+++	+	+++	+++	+++	+++
V. metschnikovii	−	+	+	+	+	−	−	−	−	−	+	++	+	++	+	−	−	−	−	−
Ps. aeruginosa	++	++	++	++	++	+	+	+	+	+	−	−	−	−	−	+++	+++	+++	+++	+++
E. coli	++	++	++	++	++	−	−	−	−	−	+++	++	++	++	++	++	+++	+	+++	+

Relative activity being recorded according to visual judgment.

* Saline water: a, Pure water; b, 0.5% NaCl solution; c, 3% NaCl solution; d, A.S.W. dilute 6-fold; e, A.S.W. (artificial sea water).

The results obtained are in Table 22. In general, Marine type bacteria were unsuitable in this experiment. Their activities were so weak that they could not be detected in indole production and nitrate reduction. However, the differences in activities between the three types M-, H-, and T-type were able to be known in the glucose decomposition and cytochrome oxidase activity.

In the T-type, for example, *Pr. vulgaris* gave indole production and nitrate reduction, and the grades of activities given were highest in 0.5 per cent NaCl medium, against the lowest result in 3 per cent NaCl medium. The activity curve also was identical to the growth curve and the active peak was at 0.5 per cent in the NaCl medium. Neither the M-type nor the H-type bacteria showed activity in the pure water medium, but showed activity in the mineral salts media. *V. parahaemolyticus* one of the H-type showed high activity of indole production and nitrate reduction in 3 per cent NaCl medium. It's activity was affected with salt concentrations. The M-type bacteria, as earlier mentioned, were known to grow in mineral salts, but they did not grow in only NaCl medium and contrasted with the H-type bacteria grew well in the same medium. The difference between the M-type and the H-type was not ascertained in the present experiment.

Biological activities that appeared in various types of bacteria were different in their types and the difference of activities between the T-type and the other two types, H- and M-type were especially distinguishable. It was assumed that the cause of such differences might be due to the differences of their own enzymic activity.

Robinson *et al.* (1952) established similar evidence. He proceeded optimally in cultures at a NaCl 2.2 per cent, concerning nitritase of *Micrococcus halodenitrificans*. In another consideration, it may be assumed that the relations between the mineral

requirement and the activity are due to not only to biochemical reactions but also to the construction of bacterial cells.

2. *Effect of various salts and their concentration on the osmotic fragility of strains selected from M-, H-, and T-type*

It is a characteristic of many marine bacteria that they lyse when placed in a hypotonic media. There are indications that the cell walls of marine bacteria may be different in nature from those of nonhalophiles (Tyler *et al.*, 1960; MacLecd *et al.*, 1962; Sud *et al.* 1963).

The author tried to compare the lysis and the fragility of strains selected from the M-, H-, and T-type bacteria tested.

Experimental methods

Lytic susceptibility. The method described by Tyler *et al.* (1960) was used with minor modifications. At first, for making the concentrated suspension, the test bacteria were transfered into the artificial sea water diluted three-fold from the plate cultures, next, one part of the suspension was dissolved in 100 parts of test solution described below. In this experiment, it was necessary that the concentration of diluted suspension was 0.5 in optical density at 630 mμ. The author ascertained the concentration of suspension by using a spectrophotometer model 4A (Tokyo Koden Co., Ltd., Japan).

The test solution used were as follows; pure water, 1.8 per cent glycerine solution, 1 per cent NaCl solution and artificial sea water diluted three fold. Then, ascertaining the concentration of suspension, the suspension was incubated at 25°C for 3 hours and again optical density of the suspension was measured. Pure water is one of hypotonic medium, and the other three were each of osmotic equivalent medium the same as the sea water diluted three-fold. The reason for selecting such concentrations was that by following the evidence that T-type bacteria growth is slowed when in the salt concentration of artificial sea water. Therefore, the sea water used for the solute was diluted to one-third of the original, and in this concentration both T-and M-type bacteria grew similarly without interference during this experiment. The media containing 0.05 per cent polypeptone and 0.01 per cent yeast extract were used, because such media were never harmful to test organisms in pure water, and also the use of such media were comparable with experiments in previous chapters. The residual turbidities percentages were calculated by taking the optical density of the control suspension in artificial sea water diluted three fold as 100 per cent residual turbidity.

Viable cell counts were made on standard pour plates using ZoBell 2216 E agar medium for marine isolates and on ordinary nutrient agar medium for terrestrial bacteria.

Experimental reults

Test organisms belonging to M-, H-, and T-type, 1055-1 and 1055-2 strains

Table 23. Lytic properties of the selected strains from each M-, H-, and T-type bacteria

Solute of test media*	Per cent residual turbidity**				
	Test strain				
	1055-1	1055-2	1007-1	*V. parahaemolyticus*	*Ps. aeruginosa*
A.S.W. diluted 3-fold	100	100	100	100	100
1% NaCl solution	70	90	80	90	95
1.8% glycerine soln.	65	90	70	80	95
pure water	65	90	75	80	90

* All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.

** Per cent residual turbidity $= \frac{\text{optical density in suspending medium}}{\text{optical density in A.S.W. diluted 3-fold}} \times 100$

Table 24. Effect of salts on viable cell counts of the selected strains from each M-, H-, and T-type

Solute of test media*	Per cent residual viable cells**				
	Test strain				
	1055-1	1055-2	1007-1	*V. parahaemolyticus*	*Ps. aeruginosa*
A.S.W. diluted 3-fold	100	100	100	100	100
1% NaCl solution	8	0	50	100	100
1.8% glycerine soln.	0	0	70	0.5	100
Pure wates	0	0	50	0.5	80

* All media (pH 7.8) contain 0.05% of polypeptone and 0.01% of yeast extract.

** Per cent residual viable cells $= \frac{\text{No. of viable cells in suspending medium}}{\text{No. of viable cells in A.S.W. diluted 3-fold}} \times 100$

were selected from M-type bacteria, 1007-1 strain and *V. parahaemolyticus* from H-type, and *Ps. aeruginosa* from T-type were used in these experiments.

The data on the lysis of bacteria and the changes of viable cell counts of the test strains grown in various test media are presented in Tables 23 and 24. As shown in Table 23, in general, the test strains were lysed only a few in various media. On the other hand, about 30 per cent residual turbidity decreased by sonication of 20 Kc for 5 min. From the result of sonicating and lysis, the author supposed that the cell lysis in the media was only partial, and a low degree of lysis was dependent on some contaminants contained in polypeptone and yeast extract in the test solution.

One of terrestrial bacteria *Ps. aeruginosa* was more or less lysed in each media. In general, most bacteria of M-type were easily lysed but like the 1055-2 strain, sometimes some of these types were not lysed in these media.

As shown in Table 24, the viable cell number of M-type bacteria decreased notable in the defined media at 25°C for 3 hours. The difference of viability between the T-type bacteria and H- or M-type bacteria was distinguishable in the experiment. In the case of the T-type bacteria, the decrease of viable cell numbers was low in all media used. That is, there was only a 20 per cent decrease in pure water and no decrease in 1 per cent NaCl solution, 1.8 per cent glycerine solution, or in artificial sea water diluted three-fold.

The viable cell numbers of *V. parahaemolyticus* belonging to the H-type did not decrease in 1 per cent NaCl solution the same as in artificial sea water diluted three-fold. That is, it generally agreed with the next, that the bacterium was able to grow in the medium containing NaCl for the sole constituent of mineral salts. Of course, in pure water, the bacterium died, also in the glycerine solution which was even isotonic compared with artificial sea water diluted three fold. 1007-1 strain from sea water like *V. parahaemolyticus* belonged to the H-type, but it seemed that the difference of both 1007-1 and *V. parahaemolyticus* was derived from their viability in various test solutions. That is, viable cell counts of 1007-1 strain decreased to 50 per cent in each 1 per cent NaCl solution or pure water, but only decreased to 70 per cent in the glycerine solution. Such characteristics as those of above bacterium could not be detected in *V. parahaemolyticus*. In other words, it means that *V. parahaemolyticus* was highly halophilic, but the 1007–1 strain was only slight halophilic. The 1007–1 strain also required the mineral salts of sea water.

Both the 1055–1 and the 1055–2 strains belonging to M-type were viable in artificial sea water diluted three-fold. And in the case of this test, most test bacteria were usually viable in artificial sea water three-fold. Viable counts of suspended bacteria were not changeable for 3 hours after incubation. After five or more hours incubation viable counts rather increased over the orginal counts. And the M-type bacteria viable in artificial sea water diluted three-fold, but they died in other media.

From the facts mentioned above, it was ascertained that the M-type bacteria have a strict requirement for mineral salts, and they were not able to grow in the medium containing NaCl for the only source of mineral salts, but they have a special requirement for NaCl. Moreover, their requirement was never replaced with the isotonic quality of other salts. In actuality, the highly specific requirement for major kinds of salts, Na-, K-, Mg-, and Ca-salt, in sea water by Marine type bacteria, and imagining the relation between these mineral requirements and the structure of the cell wall is very interesting. These are the subject of continuing investigation.

General Discussion and Conclusion

Only recently has intensive investigation been initiated with marine bacteria

concerning those properties which distinguish them physiologically from terrestrial bacteria. Even now, the criterion suggested for separating the marine species of microorganisms can not be considered sufficient.

ZoBell (1946) summarized much of the research done to that date, showing that the ability to grow in and the requirement for sea water as a base for media characterized these bacteria. MacLeod and Onofrey (1956, 1957) found that at least one major group of marine bacteria may be distinctly characterized by an absolute requirement for Na^+. On the other hand, Kriss (1963) had the view that the specific features of the sea and the ocean as the environment for microorganisms is by no means determined only by the salinity of the water. And he writes "The most hopeful criterion for determining if a given microbial form is a marine microorganisms could be its ability to reproduce in the sea. By this is meant precisely in the sea, not in isolated samples of sea water where the conditions are very different from the natural ones. An index of this ability is the frequency of the occurrence of this form, particulary when it is found in the regions of the sea or ocean at great distances from one another. An even more definite indication of reproduction in the sea is the finding of a microbial form in a water sample in greater numbers."

However, it is to be desired that a given bacterium was determined by a laboratorial method whether a marine species or not.

The author has tried to establish the easiest and most useful criterion for distinguishing marine bacteria and halophilic or terrestrial ones, and the results in the present investigation supports respectively the views of ZoBell (1946) and MacLeod and Onofrey (1956, 1957) mentioned above. However, he found that application of the views as a criterion for separating marine species of microorganisms met with a difficulty, that is difficulty in distinguishing between marine bacteria and halophilic ones. That is, according to their definition, halophilic bacteria which differed materially from the marine bacteria were dealt with as marine bacteria.

The author established in the five types of defined media for distinguishing of microorganisms, and the microorganisms are grouped to the three, Terrestrial, Halophilic, and Marine type bacteria. Among the three types, Marine type bacteria had special requirements for minerals. Not only NaCl but also the other minerals (K-, Mg-, and Ca-salt) of sea water are needed for their growth. This requirement for Na^+ or for sea water could not be replaced wholly or to any significant extent in part, either by any one of a number of related inorganic ions or by organic compounds added to increase the osmotic pressure of the medium.

On the stability of the Na^+ requirement of marine bacteria, Pratt and Waddell (1959) reported the selection of what appeared to be mutants of a marine bacterium in 1 per cent trypticase medium prepared without added NaCl, but containing the other ions of sea water. It is caused by plating heavy suspension of marine bacteria on trypticase medium containing 0.028M Na^+ present as a contaminant. MacLeod and Onofrey (1963) indicated that the Na^+ requirement of

the marine bacteria examined is a very stable one. The author also failed to train Marine type bacteria to grow in lowered artificial sea water or the other salts except NaCl in artificial sea water as mentioned in Figures 3 to 6. Therefore, it was indicated that not only the Na^+ requirement but also the requirement of the other minerals except NaCl in artificial sea water by the Marine type bacteria is a stable one. From the findings shown here, it was judged that the possession of this highly specific and stable requirement for sea water is entitled to true marine bacteria, and only the Marine type bacteria in the three types grouped by the author should be designated true marine bacteria. They could best be distinguished from land contaminants present in sea water by their growth capacity manifested in the five types of defined media mentioned here.

Next, the original habitat of *V. parahaemolyticus*, being considered to be responsible for most food poisoning caused mainly by fishes and shells had not been known and only within recent years has there been extensive studies on this subject. Miyamoto *et al.* (1960, 1961, 1962) found *V. parahaemolyticus* strains each possessing antigenic types identical with those of the food poisoning causing types, serotype XIII and XXI, respectively, at the fifth oceanic research of Sagami and Tokyo Bays in summer season of 1960. And they reported that the confirmating of the marine origin of the microorganisms are of great significance. The oceanic research by them carried out in the coastal region, one entertained a doubt, to assume the isolates from the sea relatively near land as marine bacteria. In this work, the author recognized a distinguishable difference between the *V. parahaemolyticus* and Marine type bacteria. That is, the former required NaCl, but the NaCl requirement of the bacteria could be replaced with other mineral salts. And then, they could grow at 37°C. In other words, *V. parahaemolyticus* only requires NaCl, and concerning the *V. parahaemolyticus*'s growth, NaCl has a function like osmolar control. The author did not find a close connection between the features of *V. parahaemolyticus* and marine environment. On the other hand the latter have special requirements for major minerals in the sea water, as stated above. From this fact, it was ascertained that *V. parahaemolyticus* did not belong to the marine bacteria group. This conclusion is substantiated by the facts that *V. parahaemolyticus* obtained in a great number from river water not mixed with sea water in Nagasaki City by Yasunaga (1964), and then Ose and Ikeda (1964) isolated *V. parahaemolyticus* from night soil, and their experiment showed the hypothesis about the biocycle of *V. parahaemolyticus* in nature as follows: Patients suffered from *V. parahaemolyticus*—→faeces contaminated by the microorganisms—→collection the contaminated night soil by car—→falling out the night soil to sea water by ship—→ contamination of sea water and the subsoil—→contamination of the fishes in sea water—→man who eats the fishes patients—→(upper cycle).

The majority of bacteria obtain energy from the food supplied. The food consumed is used for two purposes: one, as a source of energy and, two, as the actual material or the "building blocks" that make up the cell. The inorganic elements

are the actual "building blocks" that go into the cell structure. All bacteria require inorganic ions for their growth. The inorganic salts are needed for the permeative control, maintenance of membrane equilibrium, and co-facter of enzymic actions by organisms.

The Marine type bacteria were shown to require relatively high concentrations of Na^+ for their optimal growth and metabolic activity; this requirement had been considered to be more than a single expression of osmotic activity since the total replacement of Na^+ with nonspecific solutes had not been successful. And then they also had need of the other inorganic salts such as K-, Mg-, and Ca-salt in sea water for their growth as stated above. In the investigation (Table 22) on the effect of NaCl and the other inorganic salts on the metabolism of intact cells of Marine type bacteria, it was revealed that NaCl have specific, positive effects on their enzymic systems, but the other salts had failed to reveal any positive effect. On the other hand, Pratt *et al.* (1960) observed that intact cells of a marine bacterium required, in addition to an osmotically effective solute, Na^+ and K^+ for maximal formation of indole. Cell-free extracts, however, required K^+ but not Na^+ for indole production; concentrations of NaCl giving optimal activity with intact cells partially inhibited the activity of cell-free extracts. The evidence thus far suggests that Na^+ has specific functions in marine bacteria in transport mechanisms through the layers enveloping the cells, and that Na^+ in concentrations required for optimal growth is inhibitory to the enzymes contained in the cells. Another investigation (Table 24) has shown that lack of the other salts (K-, Mg-, and Ca-salt) accelerated fragility of the cells of Marine type bacteria in hypotonic medium or even in isotonic NaCl and glycerine medium. As cytological effect of Mg^{++} and Ca^{++} for *Rhizobium* was reported Vincent *et al.* (1962), it was revealed that the other salts such as K-, Mg-, and Ca-salt had cytological effects on their cell structure, particularly structure of cell wall, of marine bacteria.

Acknowledgements

The author wishes to express his sincere thanks to Dr. Minoru Sakai, Professor of microbiology of the Faculty of Fisheries, Hokkaido University, who not only made available to the author for the conduction of this study, his laboratory in Hokkaido University, but also kindly supplied the strains of marine bacteria stored in his laboratory and gave sympathetic guidance throughout this study. Thanks are also due to Messrs. Takahisa Kimura and Haruo Shinano for their many helpful discussions.

The author is grateful to Dr. Daiichi Kakimoto, Professor of microbiology of the Faculty of Fisheries, Kagoshima University for his constant encouragement and valuable advice during the coruse of this work.

Furthermore, thanks are also due to Prof. Dr. Katsuji Yoshimura, Prof. Dr.

Eiichi Tanikawa and Prof. Dr. Sigeru Motoda, Faculty of Fisheries, Hokkaido University, for their kindness in reading and correcting of this manuscript. And also to Mr. Richard Hendrickson for his correction of the English manuscript.

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