Mineral Requirements and other Characters of Selected Marine Bacteria

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SUMMARY

Marine bacteria from Atlantic coastal waters off Florida, isolated by a technique assuring that all would grow on a sea-water medium but not on a distilled water medium, were studied; 96 of 100 original isolates were characterized by morphological and some physiological properties, particularly those related to mineral responses. All isolates were Gram-negative rods or spiral forms, grew well in a simple saline-peptone medium, and most were pigmented and motile. All isolates required NaCl for good growth, and groupings could be clearly established for those whose mineral nutrition was satisfied, in a casein digest broth, by adding NaCl only, NaCl+KCl, NaCl+Mg salts, or all three salts. Lytic susceptibility in various mineral solutions and growth tolerance to various salt levels were studied. Some overall similarities, and a few correlations between mineral nutrition and physiological versatility, were established.

INTRODUCTION

Only recently has intensive investigation been initiated with marine bacteria concerning those properties which distinguish them physiologically from terrestrial bacteria. ZoBell (1946) summarized much of the research done to that date, showing that the ability to grow in and requirement for sea water as a base for media characterized these bacteria; solution mixtures of the major mineral components were shown to be substitutes for natural sea water for many isolates. Studies of mineral nutrition and osmotic relations have been reported for several marine bacteria (MacLeod, Onofrey, & Norris, 1954; Pratt, Boring, & Riley, 1954). The investigations reported here deal with the isolation and effects of major sea-water minerals on 96 stenohaline marine bacteria.

Studies with marine bacteria (ZoBell, 1946) and halophilic bacteria (Flannery, 1956) have shed relatively little light on the detailed mineral requirements of the former group; somewhat more information was available concerning the bacteria isolated from salt-preserved material (e.g. Robinson & Gibbons, 1952; Ingram, 1957). Luminescent bacteria, from marine sources, have been shown to be susceptible to lysis in particular salt concentrations (Johnson & Harvey, 1937; Johnson & Gray, 1949) and physiologically responsive to varied salts (Johnson & Harvey, 1938). However, the highly halophilic bacteria differ materially from the marine bacteria described by ZoBell & Upham (1944) and the restricted groups studied by us and by MacLeod *et al.* (1954).

Our results support the view of MacLeod & Onofrey (1956, 1957) that at least one major group of marine bacteria may be distinctly characterized by an absolute requirement for sodium ions (see also Ingram, 1957). Our studies have extended this observation to nearly 100 isolates (MacLeod *et al.*, detailed the requirement for six of 15 marine forms) and clearly demonstrated that requirements for other major sea-water minerals varied among the isolates and could be used to segregate them into groups; within these groups, a few correlations were found with other physiological properties.

METHODS

Basal media. The basal medium used for isolation, and for all routine cultural, morphological, and physiological tests consisted of Trypticase (Baltimore Biological Laboratory, Baltimore, Md., U.S.A.), a pancreatic digest of casein, 1 % (w/v) in sea water; 2 % (w/v) agar was used for solid medium. Modifications for certain physiological tests are described below. Special experiments concerning mineral requirements and salt tolerance necessitated use of media containing 0.1 % (w/v) trypticase, and an artificial sea water (A.S.W.) composed of reagent grades of (% w/v) NaCl, 2.4 (c. 0.4 M); KCl, 0.07 (c. 0.01 M); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.53 (c. 0.026 M); and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.70 (c. 0.028 M). The reaction of all media, without adjustment, was pH 6.9–7.2.

Mineral requirements. Preliminary experiments, confirmed by later data, demonstrated that all of the isolates grew satisfactorily on the trypticase-A.S.W. medium; neither sea water, nor complex mixtures of trace minerals were required. Initially, test media consisted of sea water (control); artificial sea-water concentrations of NaCl, NaCl + KCl, NaCl + MgCl_2 + MgSO_4 , KCl + MgCl_2 + MgSO_4 , or all the minerals; and distilled water; each with 1 % (w/v) trypticase. In later tests, the trypticase was used at 0.1 % (w/v) concentration, sea-water medium was omitted, and a test medium was added in which the only salt component was NaCl (0.6 M) at the concentration of the total salt content of sea water. Inoculum was washed from an 18 to 36 hr. culture on 0.1 % trypticase-A.S.W. agar with sterile 0.4 M-NaCl solution; 0.1 ml. of the dilute suspension (adjusted to O.D. 0.30 ± 0.05) was added to 30 ml. of test medium in 250 ml. Erlenmeyer flasks. Growth in test media was estimated by reading optical density at 500 m μ in a 'Spectronic 20' spectrophotometer (Bausch and Lomb Optical Co., Rochester, New York, U.S.A.). Precautions were taken to minimize mineral contamination: solutions were prepared with distilled water, and glassware was washed with near-neutral detergent, followed by thorough rinsing with distilled water; test cultures were incubated with stainless steel caps. In A.S.W. media, the trypticase was separately sterilized and added aseptically to sterile mineral solutions to avoid precipitation.

Characterization tests. Various tests to characterize the general morphology and cultural features of the isolates were performed according to standard techniques (*Manual of Microbiological Methods*, 1957). Colonial appearance and pigmentation were observed and recorded after 24–36 hr. at 27° on basal agar medium. Morphology was determined from smears prepared by the agar block technique, fixed in Bouin's solution, and stained by Burke's modification of the Gram stain. Physiological tests were performed in basal broth or agar to which was added the test substrate when necessary, the cultures being incubated statically at 27° for 12–36 hr. Carbohydrates were separately sterilized and added aseptically to sterile broth; 0.02 % (w/v) each of L-cystine and ferric ammonium citrate were used as

supplements in tests for production of H_2S and indole. Tests for gelatin and starch hydrolysis and for nitrate reduction to nitrite or N_2 were performed conventionally.

Lytic susceptibility. The tendency of the isolates to lyse in various mineral solutions was tested by the method of Pratt & Riley (1955) with minor modifications. A concentrated suspension of test bacteria, subsequently adjusted to give an optical density of *c.* 0.80 at 500 $m\mu$ in a Coleman Junior spectrophotometer (Coleman Instruments, Inc., Maywood, Illinois, U.S.A.) when diluted 1:100 in sea water, was harvested with sea water from basal agar plate cultures; with a few agar-digesting isolates, the cells were centrifuged from shaken, broth cultures and resuspended in sea water. The adjusted suspension was added, 0.1 ml. to 10 ml. of each of the test solutions in optically matched tubes, dispersed by inversion, and the tubes were incubated in a water bath at 37° for 15 min. The optical density of each suspension and of a control suspension in sea water was then read in the spectrophotometer, the residual turbidity of tests being referred to the control as 100%. Test solutions were distilled water and 0.50 M and 0.05 M solutions each of NaCl, KCl, and $MgCl_2$.

Salt tolerance. The ability of 15 selected isolates to multiply in salt concentrations different from that in sea water was tested in shaken broth cultures in 0.1% (w/v) trypticase-A.S.W., with NaCl-concentrations of 0.2, 0.4, 0.8, 1.4, 2.0 and 2.6 M, the other minerals being added at A.S.W. levels. Inocula were prepared and used as described for the mineral requirement tests. The shaken cultures, incubated at 30°–32°, were observed at 24 and 48 hr., relative growth being recorded according to visual judgment.

RESULTS

Isolation of cultures. A sample of water was taken several yards offshore on Florida's Atlantic coast at Matanzas Beach and another at Crescent Beach, distant from the mouth of any freshwater stream. The water was transported immediately to the laboratory and 0.1 ml. samples, six from the Matanzas Beach water and four from the Crescent Beach water, were spread on the surface of basal agar plates. When colonies developed, after incubation at 27° for 24–36 hr., replica plates (Lederberg & Lederberg, 1952) were made on basal (sea water) agar and on distilled water-trypticase agar. Isolates were selected from the replica plates after incubation, with choice being made from sea-water plates of colonies which had no replicas on the distilled water medium. Of 100 isolates thus selected, numbered M.B. 1 to M.B. 100, 96 were used in this study.

General characterization. Tests of morphological and physiological properties were inadequate to classify the isolates, but sufficed to serve as pragmatic means of distinction among them. Detailed data are not reported here, but certain of the observations are summarized. A majority of the isolates (57) formed pigment, most of them producing a cream to yellow pigment which developed slowly and became pronounced after several days' incubation. One (M.B. 66) produced a bright red, water-insoluble pigment. All cultures were Gram-negative rods or, in 14 instances, vibrio to spiral forms. Only 41 fermented one or more carbohydrates, and 42 hydrolysed starch. However, 76 hydrolysed gelatin and 72 were motile. Nitrite, but no gas, was produced from nitrate by 29 isolates, 11 produced H_2S , and 14 produced indole.

Minerals required for good growth. Preliminary tests showed that the 96 isolates all grew satisfactorily in 0.1% (w/v) trypticase broth or agar containing a mixture

of the major minerals of sea water. Each was inoculated, therefore, into tubes containing sterile 0.1 % (w/v) trypticase broth containing various combinations of salts: all components of A.S.W.; NaCl and KCl; NaCl, MgCl_2 and MgSO_4 ; KCl, MgCl_2 and MgSO_4 ; and NaCl only; each salt at the concentration in sea water (see Methods). An additional test medium contained, as the sole added salt, NaCl at 0.6 M, approximating the total salt content of sea water. Since previous tests had shown that growth in media without added NaCl was nil or negligible, inocula were prepared by suspension in 0.4 M-NaCl of the growth from 18 to 36 hr. cultures on 0.1 % trypticase-A.S.W. agar; 0.1 ml. was added to 30 ml. of test medium (300-fold dilution).

Results of these tests (Tables 1-4) demonstrated the clear-cut discriminations observable with the majority of cultures; four mineral requirement groups were obvious, as had previously been suggested by Waddell (1956). Thirty-five isolates grew significantly only when all four salts were present (Table 1); most of them grew prolifically. Only one (M.B. 59) showed slight turbidity in the presence of added NaCl only. Seventeen isolates grew sufficiently to give a turbidimetric measurement when only NaCl and magnesium salts were added (Table 2); in three instances, some growth occurred when NaCl only was added at the higher concentration. Twenty-five isolates grew adequately with addition of NaCl and KCl only (Table 3), in most instances as well as or better than in the A.S.W. medium. With seven of this group, very slight growth occurred either in the sodium-magnesium medium, or in that containing NaCl only. With the remaining 19 cultures (Table 4), growth in the medium containing added NaCl only was appreciable, and generally these isolates grew in any salt combination of which NaCl was a component; two isolates failed to produce measurable turbidity in the sodium + magnesium salt or sodium + potassium salt combinations, the reason not being apparent from the experimental data. Only four of the 96 isolates grew to measurable turbidity in the medium to which no NaCl was added (K, Mg medium), and in each instance the growth was appreciably less than in any of the media containing NaCl.

Lytic susceptibility. The sensitivity of marine isolates to lowered osmotic pressure and to particular salt solutions resulting in lysis was reported by Pratt & Riley (1955) for the selected types studied in this laboratory. The quantitative variability of results among the 96 isolates in the various test solutions made interpretation difficult; the results (Tables 1-4) are recorded according to an arbitrary classification, based on maintenance of at least 90 % turbidity in test solutions as compared to the control: 1, all test solutions; 2, none; 3, 0.5 M- MgCl_2 only; 4, 0.5 and 0.05 M- MgCl_2 only; 5, 0.5 M-NaCl, 0.5 and 0.05 M- MgCl_2 ; and 6, 0.5 M-NaCl, KCl, and MgCl_2 , and 0.05 M- MgCl_2 .

It was evident that the majority of cultures were susceptible to loss of optical density in distilled water and in 0.1 M and 0.5 M-KCl. NaCl and MgCl_2 at 0.5 M, and MgCl_2 at 0.05 M, however, provided protection to a majority of the isolates. These data do not reveal the response of isolates in terms of viability, nor are certain other equivocal aspects evident. These are the subject of continuing investigation.

Salt tolerance. Some attempts have been made (Flannery, 1956; Ingram, 1957), without marked success, to classify marine and halophilic bacteria together (or in some instances, apart) on the basis of ability to grow or survive in various levels of salt. Consequently, 15 isolates, selected to represent the four mineral requirement groups, were studied by cultivation in shaken broth cultures, the media being

Table 1. Growth of marine isolates requiring all major minerals of sea water

Isolate no.	M.B.	A.S.W.	o.d. in 0.1 % trypticase broth with indicated minerals added*								Lytic prop.†	Fermentations‡						Other activity				
			Na, Mg	Na, K	Mg, K	0.4 M NaCl	0.6 M NaCl	None	Glucose	Xylose		Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H ₂ S	Indole		
4		1.00	0	0	0	0	0	0	0	4	—	—	—	—	—	—	+	+	—	—	—	
6		1.10	0	0	0	0	0	0	0	3	—	—	—	—	—	—	—	+	—	—	—	
8		0.95	0	0	0	0	0	0	0	3	—	—	—	+	—	—	—	+	—	—	—	
10		0.66	0	0	0	0	0	0	0	5	—	—	—	—	—	—	+	+	—	—	—	
11		1.80	0	0	0	0	0	0	0	3	—	—	—	—	—	—	—	+	—	—	—	
15		1.10	0	0	0	0	0	0	0	3	—	—	—	—	—	—	—	+	—	—	—	
17		1.10	0	0	0	0	0	0	0	3	—	—	—	—	—	—	+	+	—	—	—	
18		0.62	0	0	0	0	0	0	0	5	—	—	—	—	—	—	—	+	—	—	—	
19		1.10	0	0	0	0	0	0	0	5	—	+	—	—	—	—	—	+	—	—	—	
23		0.92	0	0	0	0	0	0	0	6	—	—	—	—	—	—	—	+	—	—	—	
26		0.80	0	0	0	0	0	0	0	4	—	—	—	—	—	—	+	+	—	—	—	
27		0.75	0	0	0	0	0	0	0	6	—	—	—	—	—	—	+	+	—	—	—	
29		0.91	0	0	0	0	0	0	0	4	—	—	—	—	—	—	+	+	—	—	—	
34		0.95	0	0	0	0	0	0	0	5	—	—	—	—	—	—	—	+	—	—	—	
35		0.76	0	0	0	0	0	0	0	4	—	—	—	—	—	—	+	+	—	—	—	
36		1.10	0	0	0	0	0	0	0	2	—	—	—	—	—	—	+	+	—	—	—	
42		0.86	0	0	0	0	0	0	0	4	—	—	—	—	—	—	—	+	—	—	—	
43		0.64	0	0	0	0	0	0	0	4	—	—	—	—	—	—	+	+	—	—	—	
44		0.97	0	0	0	0	0	0	0	4	—	—	—	—	—	—	—	+	—	—	—	
45		1.10	0	0	0	0	0	0	0	4	—	—	—	—	—	—	+	+	—	—	—	
51		0.27	0	0	0	0	0	0	0	5	—	—	—	—	—	+	—	—	—	—	—	
52		0.35	0	0	0	0	0	0	0	3	—	—	+	—	—	—	—	+	+	—	—	
59		1.00	0	0	0	0.08	0.06	0	0	5	—	—	—	—	—	—	—	+	—	+	—	
62		1.10	0	0	0	0	0	0	0	5	—	—	—	—	—	—	+	+	—	—	—	
64		0.55	0	0	0	0	0	0	0	6	—	—	—	—	—	—	+	+	—	—	—	
69		1.00	0	0	0	0	0	0	0	5	—	—	—	—	—	—	—	+	—	—	—	
77		0.76	0	0	0	0	0	0	0	5	—	—	—	—	—	—	+	+	+	—	—	
78		0.35	0	0	0	0	0	0	0	5	—	—	—	—	—	—	—	—	—	—	—	
87		0.31	0	0	0	0	0	0	0	1	—	—	—	—	—	—	—	+	+	—	—	
91		0.38	0	0	0	0	0	0	0	5	—	—	—	—	—	—	—	+	+	—	—	
92		0.48	0	0	0	0	0	0	0	1	—	—	—	—	—	—	—	+	—	—	—	
93		0.36	0	0	0	0	0	0	0	3	—	—	—	—	—	—	—	+	—	—	—	
94		0.17	0	0	0	0	0	0	0	4	—	—	—	—	—	—	+	+	—	—	—	
97		0.67	0	0	0	0	0	0	0	2	—	—	—	—	—	—	+	+	—	—	—	
98		0.90	0	0	0	0	0	0	0	2	—	—	—	—	—	—	+	+	—	—	—	

* Measured in spectrophotometer at 500 m μ . Medium contained indicated minerals as NaCl, KCl, and MgCl₂ + MgSO₄. Incubated on shaker at 30° for 12–24 hr. (until maximum turbidity was reached).

† Lytic property: o.d. $\geq$ 90 % of s.w. control in: 1, all solutions; 2, no solutions; 3, 0.5 M-MgCl₂ only; 4, 0.5 M and 0.05 M-MgCl₂ only; 5, 0.5 M-NaCl, 0.5 and 0.05 M-MgCl₂; 6, 0.5 M-NaCl, KCl and MgCl₂ and 0.05 M-MgCl₂.

‡ + = produced acid in statically incubated broth tubes; — = no acidity.

0.1 % (w/v) trypticase made with solutions of the minerals of artificial sea water, in which the NaCl concentration was adjusted to 0.2, 0.4, 0.8, 1.4, 2.0, or 2.6 M. Failure to grow was judged by the absence of visible turbidity in 48 hr. at 30°–32°. The data are shown in Table 5.

It was apparent that some of the isolates tested could not multiply in the medium containing 2.0 M-NaCl, and none grew in 2.6 M-NaCl medium. All 15 isolates tested

Table 2. *Growth of marine isolates requiring sodium and magnesium salts*

Isolate no. M.B.	A.S.W.	O.D. in 0.1 % trypticase broth with indicated minerals added*							Lytic prop.†	Fermentations‡						Other activity				
		Na, Mg	Na, K	Mg, K	0.4 M- NaCl	0.6 M- NaCl	None	Glucose		Xylose	Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H ₂ S	Indole	
7	0.90	0.53	0	0	0	0	0	1	—	—	—	—	—	—	—	—	—	—		
16	1.05	0.52	0	0	0	0	0	1	—	—	—	—	—	—	—	—	—	—		
24	0.87	0.20§	0	0	0	0	0	5	—	—	+	—	—	—	—	—	—	—		
31	0.30§	0.05§	0	0	0	0	0	4	—	—	—	—	—	—	—	—	—	—		
33	1.30	0.26	0	0	0	0	0	5	—	—	—	—	—	—	—	—	—	—		
38	0.62	0.33	0	0	0	0	0	1	—	—	—	—	—	—	—	—	—	—		
50	1.40	0.41	0	0	0	0	0	5	—	—	—	—	—	—	—	—	—	—		
54	0.04	0.11	0	0	0	0	0	1	—	—	—	+	—	—	—	—	—	—		
65	1.00	0.34	0	0	0	0	0	1	—	—	—	—	—	—	—	—	—	—		
67	0.81	0.29	0	0	0	0	0	5	—	—	—	—	—	—	—	—	—	—		
71	0.46	0.20	0	0	0	0	0	5	—	—	—	—	—	—	—	—	—	—		
80	0.26	0.08	0	0	0	0	0	5	—	—	—	—	—	—	—	—	—	—		
81	0.27	0.28	0	0	0	0	0	3	+	—	—	—	—	—	+	—	—	—		
89	0.37	0.12	0	0	0	0	0	5	—	—	+	—	—	—	—	—	—	—		
48	0.95	0.29	0	0	0	0.07	0.07	5	—	—	—	—	—	—	—	—	—	—		
83	1.20	0.43	0	0	0	0.20	0	5	—	—	—	—	—	—	—	—	—	—		
84	1.00	0.35	0	0	0	0.23	0	5	—	—	—	—	—	—	—	—	—	—		

*, †, ‡. See Table 1.

§ Growth very flaky-gelatinous, probably greater than indicated by 0.1

Table 3. *Growth of marine isolates requiring sodium and potassium salts*

Isolate no. M.B.	A.S.W.	O.D. in 0.1 % trypticase broth with indicated minerals added*							Lytic prop.†	Fermentations‡						Other activity				
		Na, Mg	Na, K	Mg, K	0.4 M- NaCl	0.6 M- NaCl	None	Glucose		Xylose	Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H ₂ S	Indole	
1	0.83	0	0.54	0	0		0	0	3	—	—	—	—	—	—	+	—	—	—	
3	0.77	0	0.75	0	0		0	0	5	+	—	—	—	—	—	+	—	—	—	
5	0.85	0	0.66	0	0		0	0	3	+	—	—	—	—	—	—	—	—	—	
9	1.20	0	0.15	0	0		0	0	5	+	—	—	—	—	—	—	—	—	—	
12	0.80	0	0.64	0	0		0	0	3	+	—	—	—	—	—	—	—	—	—	
14	0.76	0	0.88	0	0		0	0	6	+	—	—	—	—	—	—	—	—	—	
20	0.78	0	0.52	0	0		0	0	5	+	—	—	—	+	—	—	—	—	—	
22	0.72	0	0.68	0	0		0	0	5	+	—	—	—	+	+	—	—	—	—	
25	0.47	0	1.10	0	0		0	0	5	+	—	—	—	+	+	—	—	—	—	
28	1.40	0	1.10	0	0		0	0	3	+	—	—	—	—	—	—	—	—	—	
39	1.20	0	0.86	0	0		0	0	5	+	—	—	—	—	—	—	—	—	—	
40	1.10	0	0.64	0	0		0	0	5	+	—	—	—	—	—	—	—	—	—	
58	0.11	0	0.12	0	0		0	0	2	—	—	—	—	—	—	—	—	—	—	
61	0.17	0	0.10	0	0		0	0	2	—	—	—	—	—	—	—	—	—	—	
76	0.37	0	0.24	0	0		0	0	5	+	+	—	—	—	—	—	—	—	—	
82	0.10	0	0.31	0	0		0	0	6	+	—	—	—	—	+	—	—	—	—	
90	0.23	0	0.43	0	0		0	0	3	+	—	—	—	—	—	—	—	—	—	
95	0.11	0	0.07	0	0		0	0	5	—	—	—	—	—	—	—	—	—	—	
30	0.41	0.04	0.42	0	0		0	0	1	+	—	—	—	—	—	+	—	—	—	
57	0.44	0	0.16	0	0.05		0	0	5	—	—	—	—	—	—	+	—	—	—	
60	0.58	0.16	0.58	0	0		0	0	5	+	—	—	—	—	—	+	—	—	—	
63	1.10	0	0.66	0	0.05		0	0	5	+	—	—	—	—	—	+	—	—	—	
68	0.75	0.13	0.24	0	0		0	0	6	—	—	—	—	—	—	—	—	—	—	
75	0.38	0.11	0.31	0	0		0	0	5	+	—	—	—	—	—	+	—	—	—	
86	0.40	0.11	0.25	0	0		0	0	5	—	—	+	—	—	—	—	+	—	—	

*, †, ‡. See Table 1.

Table 4. *Growth of marine isolates requiring sodium salts only*

Isolate no. M.B.	A.S.W.	O.D. in 0.1 % trypticase broth with indicated minerals added*						Lytic prop.†	Fermentations‡						Other activity				
		Na, Mg	Na, K	Mg, K	0.4 M- NaCl	0.6 M- NaCl	None		Glucose	Xylose	Maltose	Lactose	Sucrose	Mannite	Starch	Gelatin	Nitrite	H ₂ S	Indole
2	0.95	0.75	0.43	0	0.28	0.16	0	2	+	-	-	-	-	-	+	+	-	-	-
13	0.82	0.36	0.90	0	0.38	0.17	0	6	+	-	+	-	-	-	+	+	+	+	+
21	0.90	0.38	0.95	0.15	0.41	0.38	0	6	-	-	+	-	+	+	-	+	+	+	+
32	0.51	0.13	0.56	0	0.20	0.03	0	5	+	-	-	-	+	+	-	+	+	+	-
37	0.89	0.19	1.00	0	0.15	0	0	2	-	-	+	-	-	-	-	+	+	+	-
41	1.00	0.54	0.70	0	0.26	0.25	0	4	-	-	-	-	-	-	-	+	-	-	-
47	0.87	0.42	0.98	0.19	0.48	0.40	0	3	-	+	-	-	-	-	-	-	-	-	-
49	0.70	0.23	0	0	0.29	0.19	0	5	+	-	-	-	-	-	+	+	-	-	-
53	0.95	0.33	1.00	0.14	0.35	0.34	0	5	-	-	-	-	-	-	-	+	-	-	-
55	0.90	0.32	0.64	0	0.18	0.14	0	2	+	-	-	-	-	-	+	+	-	-	-
56	0.68	0.05	0.56	0	0.10	0	0	5	-	-	+	-	-	-	-	-	-	-	-
66§	0.35	0.36	0.23	0	0.23	0.30	0	5	+	+	-	-	+	+	+	+	-	-	-
72	0.30	0.14	0	0	0.20	0	0	4	+	-	+	-	-	+	+	+	+	-	+
73	0.75	0.19	0.60	0	0.31	0.19	0	5	+	-	+	-	-	-	+	+	-	-	-
74	0.45	0	0.35	0	0.14	0	0	5	+	-	+	-	-	-	+	+	-	-	-
88	0.37	0.16	0.17	0	0.13	0.05	0	5	-	+	-	-	-	-	-	-	-	-	-
96	0.29	0	0.23	0	0.11	0	0	6	+	-	+	-	-	-	+	+	+	-	-
99	0.92	0.50	0.95	0.08	0.56	0.49	0	6	+	-	+	-	+	+	+	+	+	-	-
100	0.50	0.20	0.58	0	0.33	0.13	0	6	+	-	+	-	+	+	+	+	+	+	-

*, †, ‡. See Table 1. § Bright red pigment (water insoluble).

grew well when the NaCl level in artificial sea water was reduced to 0.2 M; six of the 15 showed no growth in NaCl levels above 0.8 M. Thus, these isolates were clearly distinct from the widely studied halophilic group whose growth has been shown to be inhibited at salt levels below about 2.0 M, yet constitute a 'halophilic' group growing optimally at salt levels near that in sea water.

Table 5. *Salt tolerance of selected marine bacteria*

Isolate no. M.B.	Relative growth* in 0.1 % trypticase broth with indicated NaCl concn. (M)					
	0.2	0.4	0.8	1.4	2.0	2.6
3	++	++	+	0	0	0
13	++	++	++	0	0	0
19	++	+++	++	++	++	0
22	++	+++	++	0	0	0
29	++	++	++	+++	+++	0
31	+	++	++	++	+	0
34	++	++	+++	++	+	0
43	+++	++	++	0	0	0
58	+	+	+	+	+	0
60	++	++	++	0	0	0
73	++	++	++	++	++	0
83	++	++	++	++	++	0
84	++	++	++	++	++	0
98	++	++	++	+++	+++	0
100	++	++	++	0	0	0

* Judged by visual turbidity in cultures incubated 48 hr. on a shaker at 30°. All media contained KCl, MgCl₂ and MgSO₄.

DISCUSSION

Two properties seem particularly to demonstrate a close interrelationship among the 96 isolates: the narrow range of salinities tolerated, and the universal requirement for Na^+ for growth. Failure of any isolate to multiply in the absence of added salt places the group in the category of obligate halophiles; yet they do not precisely fit the classifications either of Flannery (1956) or of Ingram (1957). In the former, they would be excluded by growing well in media with less than 0.3 M-salt, and in the latter, they would be included with a group (IIIA) characterized by growth in media containing salt up to well above half-saturation. All of the isolates required some NaCl in the medium to grow well; preliminary experiments have shown that some, but not all, of the NaCl requirement may be replaced by an osmotic substitute such as sucrose. This requirement for Na^+ at more than trace levels may be a distinctive property of these marine bacteria (see MacLeod & Onofrey, 1957).

Certain correlations of properties became apparent when the isolates were grouped on the basis of mineral requirements. Carbohydrate fermentation was observed with 19 of the 25 isolates which required only $\text{Na}^+ + \text{K}^+$ and with 17 of the 19 which grew well with Na^+ as the only added cation. In contrast, only eight of the 52 isolates showing a specific requirement for Mg^{++} fermented one or two of the substrates tested by the technique used. Similarly distributed was the capacity to produce nitrite from nitrate: 17 of the $\text{Na}^+ + \text{K}^+$, and eight of the Na^+ group possessed this ability, while only four of the remaining 52 isolates produced nitrite. Indole production was limited to members of the $\text{Na}^+ + \text{K}^+$, and Na^+ groups, 11 occurring in the former, and three in the latter. H_2S production, occurring with only 11 isolates, was also restricted largely to the $\text{Na}^+ + \text{K}^+$ and Na^+ groups, with only one isolate showing this property among the other groups. These observations suggest that physiological versatility is a more common feature of marine isolates whose mineral nutrition can be satisfied by adding only Na^+ , or $\text{Na}^+ + \text{K}^+$ than of those requiring Mg^{++} as well. At the same time, it must be noted that some representatives of the 'versatile' group (e.g. M.B. 58, M.B. 95) were as limited physiologically, according to these criteria, as any members of the other group.

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REFERENCES

- FLANNERY, W. L. (1956). Current status of knowledge of halophilic bacteria. *Bact. Rev.* 20, 49.
- INGRAM, M. (1957). Micro-organisms resisting high concentrations of sugars or salts. In *Microbial Ecology, Symp. Soc. gen. Microbiol.* 7, 90.
- JOHNSON, F. H. & GRAY, D. H. (1949). Nuclei and large bodies of luminous bacteria in relation to salt concentration, osmotic pressure, temperature, and urethane. *J. Bact.* 58, 675.
- JOHNSON, F. H. & HARVEY, E. N. (1937). The osmotic and surface properties of marine luminous bacteria. *J. cell. comp. Physiol.* 9, 363.
- JOHNSON, F. H. & HARVEY, E. N. (1938). Bacterial luminescence, respiration, and viability in relation to osmotic pressure and specific salts of the sea. *J. cell. comp. Physiol.* 11, 213.

- LEDERBERG, J. & LEDERBERG, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63**, 399.
- MACLEOD, R. A. & ONOFREY, E. (1956). Nutrition and metabolism of marine bacteria. II. Observations on the relation of sea water to the growth of marine bacteria. *J. Bact.* **71**, 661.
- MACLEOD, R. A. & ONOFREY, E. (1957). Nutrition and metabolism of marine bacteria III. The relation of sodium and potassium to growth. *J. cell. comp. Physiol.* **50**, 389.
- MACLEOD, R. A., ONOFREY, E. & NORRIS, M. E. (1954). Nutrition and metabolism of marine bacteria. I. Survey of nutritional requirements. *J. Bact.* **68**, 680.
- Manual of Microbiological Methods* (1957). New York, U.S.A.: McGraw-Hill Book Co.
- PRATT, D. B., BORING, J. R. III & RILEY, W. H. (1954). Regulation of sodium chloride tolerance of marine bacteria by potassium ion concentration. *Bull. Amer. Inst. Biol. Sci.* **4**, 68.
- PRATT, D. B. & RILEY, W. H. (1955). Lysis of a marine bacterium in salt solutions. *Bact. Proc.* **55**, 26.
- ROBINSON, J. & GIBBONS, N. E. (1952). The effects of salts on the growth of *Micrococcus halodenitrificans* n.sp. *Canad. J. Bot.* **30**, 155.
- WADDELL, G. H. (1956). *Marine bacteria: adaptation to non-marine environments*. Thesis, University of Florida, Gainesville, Florida, U.S.A.
- ZOBELL, C. E. (1946). *Marine Microbiology*. Waltham, Massachusetts, U.S.A.: Chronica Botanica Co.
- ZOBELL, C. E. & UPHAM, H. C. (1944). A list of marine bacteria including descriptions of sixty new species. *Bull. Scripps Inst. Oceanogr.* **5**, 239.