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Effect of Sodium Chloride on Growth of Heterotrophic Marine Bacteria

John L. Reichelt and Paul Baumann

Department of Bacteriology, University of California, Davis, California 95616

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Abstract. The effect of NaCl on the growth rates and yields of 31 gram-negative, heterotrophic, marine bacteria was determined. The strains used were representative of aerobic genera (*Alteromonas, Pseudomonas, Alcaligenes, Bdellovibrio*) as well as genera comprised of facultative anaerobes (*Beneckea, Photobacterium*). Two media were used—the first, a medium designed for the cultivation of marine bacteria and, the second, a medium used for the cultivation of terrestrial strains. These two media differed in the concentrations of divalent cations; the terrestrial medium (TM) contained 2 mM Mg^{++} and 0.55 mM Ca^{++} while the marine medium (MM) contained 50 mM Mg^{++} and 10 mM Ca^{++}. The amount of NaCl necessary for optimal growth varied in different strains and was usually considerably higher in TM (100 to 460 mM) than in MM (70 to 300 mM). Many strains which grew in MM and TM had a shorter generation time in the former than in the latter medium. In addition, four strains which grew well in MM usually failed to grow in TM. These results show that higher levels of divalent cations are either essential for growth or stimulate growth rate, indicating that for many marine strains a terrestrial medium modified by the addition of NaCl cannot support optimal growth. Fourteen terrestrial strains of the genera *Pseudomonas, Alcaligenes, Acinetobacter, Salmonella, Aeromonas*, and *Vibrio* did not have ionic requirements comparable to those of the marine strains. All of the terrestrial organisms grew in TM without added NaCl (0.068 mM Na^{+} was present as a contaminant). In some terrestrial organisms, growth was stimulated by the addition of NaCl, the highest stimulation being found in *Vibrio cholerae*. The optimal growth rates and yields for four strains of this species were observed at 2.5 to 5.0 mM NaCl while the growth rates and yields in TM with no added NaCl were 40 to 50% of the optimum.

Key words: Sodium Chloride — Growth — Marine Bacteria.

A large proportion of the bacterial flora of the oceans consists of gram-negative organisms which have a specific requirement for sodium ion [see MacLeod (1965, 1968) for reviews]. MacLeod (1968) has devised two defined media which test for the presence of this requirement. The first medium contains an artificial sea water base, while the second is modified by a replacement of sodium by equimolar amounts of potassium. Using

Non-Standard Abbreviations. ATCC = American Type Culture Collection; GC = guanine plus cytosine; MM = marine medium; TM = terrestrial medium.

these media it has been shown that 700 strains of marine bacteria from 37 distinct species and groups of the genera *Beneckea*, *Photobacterium*, *Alteromonas*, *Pseudomonas*, and *Alcaligenes* (Baumann *et al.*, 1971a, b, 1972, 1973; Reichelt and Baumann, 1973 a, b) grow only in the presence of sodium. A requirement for sea water or for 0.34 to 0.51 M NaCl for optimal growth has also been demonstrated in marine isolates of *Desulfovibrio* (Trüper *et al.*, 1969), green and purple sulfur bacteria (Matheron and Baulaigue, 1972; Trüper, 1968), caulobacters (Poindexter, 1964), agar decomposers (Stanier, 1941), spirilla (Williams and Rittenberg, 1957), nitrifying bacteria (Watson, 1965, 1971; Watson and Waterbury, 1971), a spirochete (Hespell and Canale-Parola, 1970), and *Leucothrix mucor* (Harold and Stanier, 1955). These marine isolates differ in the extent of their sodium requirement from the moderate halophiles such as *Vibrio costicolus* which requires about 1 M NaCl for optimal growth (Forsyth and Kushner, 1970) and from the extreme halophiles which require 3.4—5.1 M (Larsen, 1962).

The basis for the sodium requirement has been extensively studied by MacLeod and his collaborators in a marine bacterium designated strain B-16 which has been characterized and identified as *Alteromonas haloplanktis* (Baumann *et al.*, 1972; Reichelt and Baumann, 1973a). In this organism, the requirement of 200 to 300 mM NaCl for optimal growth reflects a specific requirement for sodium by a number of essential cellular processes, including the maintenance of the integrity of the cell wall and cell membrane, the retention of solutes, and the operation of permease systems for a variety of organic and inorganic substrates (Forsberg *et al.*, 1970; MacLeod, 1965, 1968; Thompson and MacLeod, 1971; Wong, 1968; Wong *et al.*, 1969). A requirement for sodium for growth was initially thought to be sufficient to distinguish gram-negative marine bacteria from gram-negative terrestrial forms (MacLeod and Onofrey, 1956, 1957). Subsequently, it has been found that several species of the latter also require sodium ion for growth; the concentrations necessary, however, are relatively low and often depend on the conditions of cultivation. *Escherichia coli* was found to require 3 mM sodium for an optimal growth rate on L-glutamate (Frank and Hopkins, 1969), *Salmonella typhimurium* requires about 8 mM for an optimal yield with citrate (O'Brien *et al.*, 1969), and *Aerobacter aerogenes* requires about 50 mM for an optimal yield in the fermentation of citrate (O'Brien and Stern, 1969). That the requirement for sodium in these terrestrial organisms is restricted to a few metabolic functions is evident from their ability to grow on a number of carbon compounds in the absence of this ion. Sistrom (1960) has found that, in a medium containing several carbon sources, *Rhodopseudomonas spheroides* requires about 2 mM sodium for optimal growth rate while a similar medium without added sodium supports the growth of *Rhodo-*

spirillum rubrum. The rumen inhabitant *Bacteroides amylophilus* has been shown to require about 60 mM sodium for an optimal growth rate and yield (Caldwell *et al.*, 1973). A requirement for sodium appears to be widespread among rumen bacteria (Bryant *et al.*, 1959; Hudson and Caldwell, 1972) and may be a reflection of the high sodium content (70—140 mM) of the rumen fluid (Emery *et al.*, 1960; Warner and Stacy, 1965).

Determinations of the effect of sodium on growth yields have been performed for a limited number of marine strains by MacLeod (1965, 1968). The present work consists of a study of the effect of sodium on the growth rates and yields of strains representative of the marine species and groups previously characterized in our laboratory as well as its effect on selected gram-negative terrestrial strains.

Methods

Biological Materials. The strain numbers of the marine bacteria refer to organisms characterized by Baumann *et al.* (1971a, 1972, 1973), Reichelt and Baumann (1973a, b), and Taylor, Baumann, Reichelt, and Allen (manuscript in preparation). *Salmonella typhimurium* suc LL was the gift of Dr. M. Herzberg. Strains of other non-marine bacteria were obtained either from the collection of the Department of Bacteriology, University of California, Berkeley, Calif., or from the American Type Culture Collection (ATCC).

Bacteriological Media. Two media were used; the first was a slight modification of the medium of Palleroni and Doudoroff (1972), used for the cultivation of terrestrial pseudomonads, while the second was a medium similar to the one of MacLeod (1968), designed for the cultivation of marine bacteria and utilized in our previous studies. The terrestrial medium (TM) contained 100 mM Tris, 12 mM succinic acid, 19 mM NH_4Cl, 10 mM KCl, 2 mM $MgSO_4$, 0.55 mM $CaCl_2$, 0.33 mM KH_2PO_4, and 50 mg/l ferric ammonium citrate. The pH of the medium was adjusted to pH 7.5 with 4 N HCl. The marine medium (MM) differed from TM in having 50 mM $MgSO_4$ and 10 mM $CaCl_2$. In all cases glass distilled water was used and the chemicals were of reagent grade. After autoclaving, sterile distilled water was added to the medium to restore it to its original volume. The levels of contaminating Na^+ in TM and MM were 0.068 and 0.107 mM, respectively, as determined by atomic absorption spectroscopy. For the cultivation of *Alteromonas macleodii*, 24 mM acetic acid was used instead of succinic acid. *Alteromonas haloplanktis* was also tested in MM in which succinic acid had been replaced by one of the following carbon compounds: D-glucose, D-galactose (8 mM), sucrose (4 mM), acetate (24 mM), citrate, or L-glutamate (12 mM). For the cultivation of host-independent, marine bdellovibrio 4, TM and MM were modified by omitting succinic acid and adding 0.3% Difco Yeast Extract and 1.0% Difco Peptone. The contaminating level of sodium in these media was 9.5 mM. *Aeromonas formicans*, *Vibrio albensis*, and strains of *Vibrio cholerae* were grown in TM in which 8 mM D-glucose was used instead of succinic acid. For *Vibrio albensis*, which had extensive growth factor requirements, the medium also contained 0.06% Difco Yeast Extract. The sodium contamination of this medium was 0.52 mM.

Growth Experiments. Strains were grown in 125 ml sidearm flasks and the turbidity was measured by means of a Klett-Summerson Colorimeter using a green filter. Marine strains in mid-logarithmic growth phase (50—80 Klett units) in MM con-

taining 200 mM NaCl were inoculated into a series of sidearm flasks containing MM or TM with various concentrations of NaCl. The 0.1 ml inoculum raised the sodium level in the media by 0.8 mM. Terrestrial strains were grown in TM containing 24 mM NaCl. Inoculation of 0.1 ml into homologous media raised the sodium level by 0.096 mM. The cultures were incubated in a reciprocating water bath shaker (110 strokes/min). After a period of 5—7 h the turbidity of the cultures was usually measured at half hour intervals until growth was completed. The growth rate (doublings per hour) and generation time (time required for the population to double) were determined during exponential phase of growth. It was essential that the turbidity representing the final growth yield be determined soon after termination of growth since in many strains of marine bacteria the turbidity dropped soon after growth ceased. Depending on the strain, a total of 6—14 generations elapsed from the time of inoculation to the completion of growth. For turbidities above 75 Klett units, corrections were made for deviations from linearity. Strains of terrestrial bacteria, all of which grew in TM in the absence of added sodium, were carried through three subcultures in such media. *Photobacterium fischeri* and marine bdellovibrio 4 were grown at 25°C; all the remaining strains were grown at 30°C. After an incubation period of 3 days at 30°C or 6 days at 25°C, less than 2% of the volume of the media was lost due to evaporation. All experiments were terminated within 3 days at 30°C and 6 days at 25°C.

Results

Marine Strains

With the exception of *Alteromonas macleodii* and marine bdellovibrio 4, all the strains were tested for their response to different NaCl concentrations in MM and TM with succinate as the sole source of carbon and energy. This compound was chosen since it is utilized by most strains and is a non-fermentable substrate. *Alteromonas macleodii*, a species unable to utilize succinate, was tested with acetate while host-independent bdellovibrio 4 was tested on a complex medium. The growth of all the strains was exponential throughout the growth phase at all sodium concentrations at which growth occurred. For any one strain, the lines of a semi-log plot, when extrapolated, intersected approximately at the same point indicating that growth was initiated at about the same time irrespective of the NaCl concentration of the medium. The lag before the initiation of growth was usually less than 1 h. The results which show the relation of relative growth rate and yield to NaCl concentration in MM and TM are presented in Figs. 1 and 2. The values have been normalized by making the highest growth rate and yield equal to 100%; the NaCl concentrations shown represent the sum of the contaminating Na^+ concentrations and the concentrations of NaCl added to the media. Of the 20 species and groups tested, 16 grew in both media while strains 51 (group B-1), 212 (group I-2), 195 (group H-1), and 207 (group I-1) grew only in MM. In the case of strains 51 and 195, growth was occasionally observed in TM at one or more of the higher NaCl concentrations (above 200 mM); however, the initiation of growth was erratic and occurred

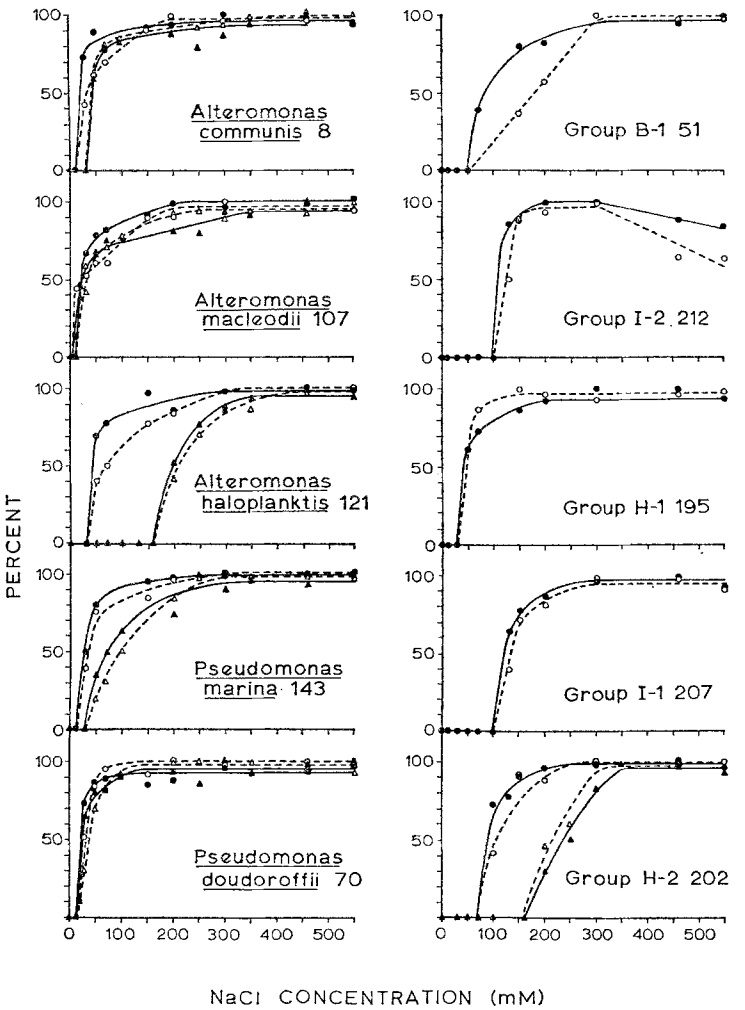

Fig. 1. Effect of NaCl on growth of marine bacteria. •——• Yield in marine medium; o- - - - o growth rate in marine medium; ▲——▲ yield in terrestrial medium; ▵- - - - ▵ growth rate in terrestrial medium. All values have been normalized by making the maximal growth rate (doublings per hour during exponential phase) and yield equal 100%

only after an extensive and variable lag period. In general, the normalized growth rates and yields of a strain in either TM or MM were similar over the range of NaCl concentrations tested (Figs. 1 and 2). A comparison of the relative growth rates and yields in MM and TM indicated that in many strains a lesser amount of NaCl was required for an equivalent

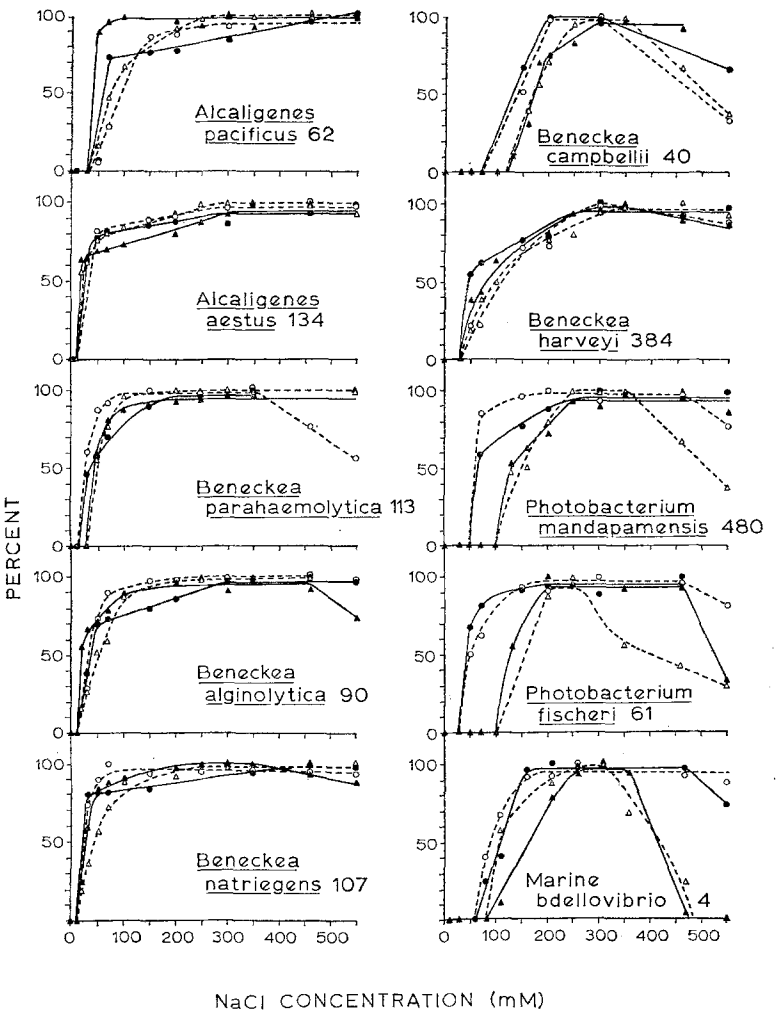

Fig. 2. Effect of NaCl on growth of marine bacteria. •——• Yield in marine medium; o- - - -o growth rate in marine medium; ▲——▲ yield in terrestrial medium; △- - - -△ growth rate in terrestrial medium. All values have been normalized by making the maximal growth rate (doublings per hour during exponential phase) and yield equal 100%

relative growth rate and yield in MM than in TM. Since MM differed from TM in having higher Mg^{++} and Ca^{++} concentrations, these results indicated that either one or both of these cations may reduce the sodium requirement. The extent of the reduction was generally slight in strains which had a relatively low requirement for NaCl (e.g. *Alteromonas com-*

munis, *Pseudomonas doudoroffii*) and extensive in those strains which required high amounts of NaCl (e.g. *Alteromonas haloplanktis*, group H-2). The reduction of the NaCl requirement by divalent cations was also indicated from the results presented in Table 1 in which the concentrations of NaCl necessary for 50% of the relative maximal growth rate in TM and MM were compared. In 13 out of the 16 strains which grew in both MM and TM, the concentrations of NaCl required for 50% of the relative optimal growth rate was 10—100 mM lower in MM than in TM. Three strains (*Alcaligenes pacificus*, *A. aestus*, *Beneckea harveyi*) required slightly more NaCl (10—15 mM) in MM than in TM. The lowest concentration of NaCl necessary for optimal growth rate and yield ranged from 100—460 mM in TM and 70—300 mM in MM (Figs. 1 and 2). The highest concentrations tested at which no growth occurred ranged from 10—160 mM in TM and 5—100 mM in MM. A similar maximal yield was observed when marine strains were grown in either MM or TM. In many strains, the maximal generation times were longer in TM than in MM (Table 1) indicating that the higher levels of Mg^{++} and Ca^{++} present in MM stimulated the growth rate. The largest difference was observed with *Alteromonas communis* (strain 8) for which the generation time in TM was 2.5 times longer than in MM. A significant exception was strain 40 of *Beneckea campbellii* which had a shorter generation time in TM than in MM although the requirement for NaCl was greater in the former than in the latter medium (Fig. 2). Strain 212 (group I-2), *Beneckea alginolytica*, *B. campbellii*, *Photobacterium mandapamensis*, and marine bdellovibrio 4 were inhibited by high NaCl concentrations (460—550 mM), the inhibition being greater in TM than in MM (Figs. 1 and 2). In the case of *Beneckea parahaemolytica* the reverse was observed; high NaCl concentrations slightly inhibited growth in MM but not in TM.

When the response of yield and growth rate to variation of the NaCl concentration in MM was tested for six additional strains (1, 6, 16, 19, 24, 32) of *Alteromonas communis*, results similar to those for strain 8 were obtained (Figs. 1 and 3). Strains 205 of *B. parahaemolytica* and 118 of *B. alginolytica* likewise gave results closely resembling those obtained with two other strains of these species (Fig. 2). In addition, four strains of *B. harveyi* (340, 352, 384, and 392) tested in MM containing D-glucose rather than succinate were found to give a response similar to that of strain 384 grown in succinate (Fig. 2). These observations suggest that strains of a single species may respond in a similar manner to variations in NaCl concentration.

The effect of different carbon and energy sources on the pattern of growth at different concentrations of NaCl was tested with strain 121 of *Alteromonas haloplanktis*. The normalized results are presented in Fig. 4 and the generation times as well as the concentrations of NaCl at which

Table 1. Comparison of the effect of NaCl on growth in MM and TM

Species or group	Moles % GC[a]	Source of carbon and energy	Concentration of NaCl (mM) allowing 50% of the maximal growth rate in		Generation time (min) at optimum NaCl concentration in		Generation time in TM/generation time in MM
			MM	TM	MM	TM	
Alteromonas communis 8	48.0	Succinate	30[b]	40	54	134	2.5
A. macleodii 107	45.9	Acetate	25	35	83	136	1.6
A. haloplanktis 121	42.9	Succinate	70	210	101	148	1.5
Pseudomonas marina 143	63.3	Succinate	30	100	120	138	1.2
P. doudoroffii 70	59.7	Succinate	30	40	54	79	1.5
Group B-1 51	63.8	Succinate	180	NG[c]	95	NG	
Group I-2 212	63.6	Succinate	125	NG	86	NG	
Group H-1 195	54.1	Succinate	50	NG	75	NG	
Group I-1 207	56.6	Succinate	130	NG	69	NG	
Group H-2 202	30.6	Succinate	110	210	53	83	1.6

Alcaligenes pacificus 62	67.9	Succinate	90	75	83	111	1.3
A. aestus 134	57.7	Succinate	30	20	92	109	1.2
Beneckea parahaemolytica 113	46.4	Succinate	25	50	78	106	1.4
B. alginolytica 90	46.4	Succinate	30	50	74	74	1.0
B. natriegens 107		Succinate	25	45	60	60	1.0
B. campbellii 40	47.2	Succinate	140	170	276	173	0.6
B. harveyi 384	46.0	Succinate	110	100	104	104	1.0
Photobacterium mandapamensis 480	42.9	Succinate	60	150	159	148	0.9
P. fischeri 61 [d]	39.0	Succinate	50	155	296	296	1.0
Marine bdellovibrio 4 [d]	38.6	Complex medium	90	110	436	534	1.2
A. haloplanktis 121	42.9	D-Glucose	70	NT [e]	81	NT	
A. haloplanktis 121	42.9	D-Galactose	45	NT	160	NT	
A. haloplanktis 121	42.9	Sucrose	70	NT	101	NT	
A. haloplanktis 121	42.9	Acetate	120	NT	148	NT	
A. haloplanktis 121	42.9	Citrate	80	NT	106	NT	
A. haloplanktis 121	42.9	L-Glutamate	45	NT	188	NT	

[a] Data from Baumann *et al.* (1971 a, 1972) and Reichelt and Baumann (1973 b).
[b] For strains 1, 6, 16, 19, 24, and 32 of this species, the amount of NaCl required for 50% of the maximal growth rate was 30 to 40 mM.
[c] NG = no growth or erratic growth pattern.
[d] Tested at 25° C.
[e] NT = not tested.

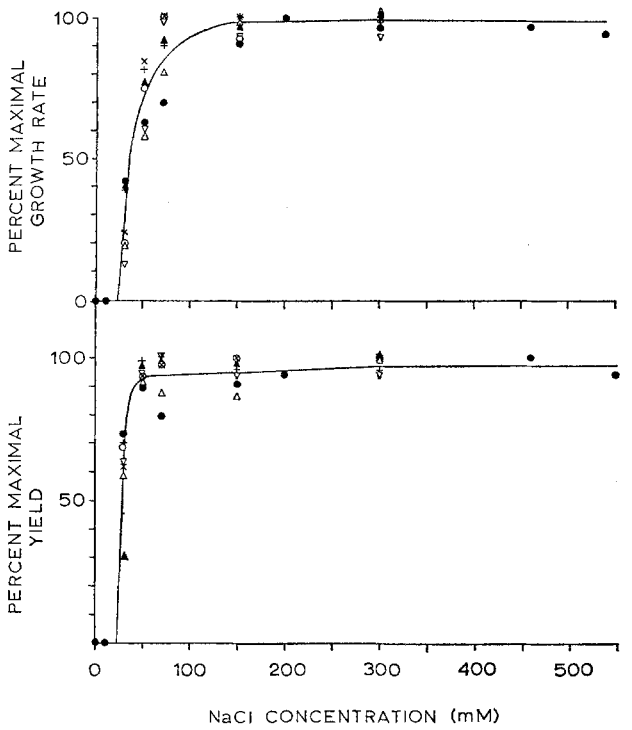

Fig. 3. Effect of NaCl on the growth rates and yields of seven strains of *Alteromonas communis*. × *A. communis* strain 1; • strain 6; ○ strain 8; ▲ strain 16; ▽ strain 19; + strain 24; △ strain 32. All values have been normalized by making the maximal growth rate (doublings per hour during exponential phase) and yield equal 100%. Line represents the mean of the data for the seven strains

the growth rates were 50% of the optimum are given in Table 1. The effect of NaCl on the relative growth rate and yield varied to some extent with the carbon source utilized and appeared to be independent of the maximal generation time. The least amount of NaCl (100 mM) was required for optimal growth on D-galactose while the highest amount (300 mM) was required for optimal growth on acetate. The generation times for both carbon sources were similar (160 min for D-galactose and 148 min for acetate). The concentration of NaCl necessary for optimal growth with D-glucose, sucrose, citrate, and L-glutamate was 200 mM. The minimal NaCl concentration at which growth occurred was also to some extent dependent on the carbon source. With D-galactose, acetate, sucrose, and L-glutamate, growth was observed at 50 mM but not at 30 mM NaCl. Some growth occurred at 30 mM NaCl with D-glucose, whereas with citrate as the carbon source, growth failed to occur even at 50 mM NaCl.

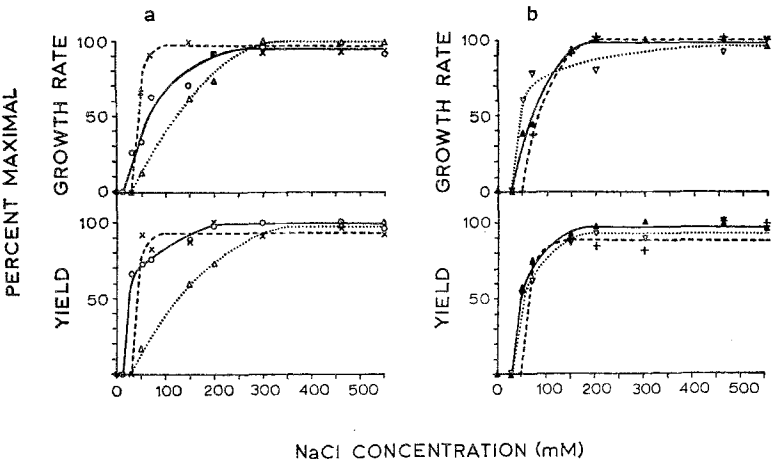

Fig. 4a and b. Effect of carbon source on the response of *Alteromonas haloplanktis* 121 to variation in NaCl concentration in marine medium. a o——o D-glucose; ×- - - -× D-galactose; ▵. . . .▵ acetate. b ▲——▲ Sucrose; +- - - -+ citrate; ▿. . . .▿ L-glutamate. All values have been normalized by making the maximal growth rate (doublings per hour exponential phase) and yield equal 100%

Terrestrial Strains

The effect of NaCl on growth rate and yield in TM for a variety of terrestrial strains is presented in Fig. 5. Most strains were not affected by the absence of added NaCl. The growth rate but not the yield of one species, *Acinetobacter calco-aceticus*, was inhibited 22% by 50 mM NaCl. In the case of *Alcaligenes faecalis*, NaCl slightly stimulated the growth rate without affecting the yield, while in *Pseudomonas fluorescens*, both the growth rate and yield were slightly stimulated by the addition of NaCl. The growth rate and yield of *Vibrio cholerae* (ATCC 14035) was reduced by 45% in TM containing no added NaCl compared to TM containing 2.5 mM NaCl, the lowest tested concentration giving optimal growth rate and yield. Three other strains of this species (ATCC 14101, 25870, 25872) gave similar results; the optimum for the growth rate and yield ranged from 2.5—5.0 mM NaCl and the growth rate and yield in TM with no added NaCl was reduced by 50—60%. In all cases, the values for the growth rate and yield in TM with no added NaCl (0.068 mM Na^+ present as a contaminant) represents the results of three serial subcultures during which no change was observed in either growth rate or yield. *Vibrio albensis* (ATCC 14547), an organism which had extensive growth factor requirements, was tested in TM containing 0.06% yeast extract (Na^+ level of 0.52 mM). When serially transferred in this medium, *Vibrio*

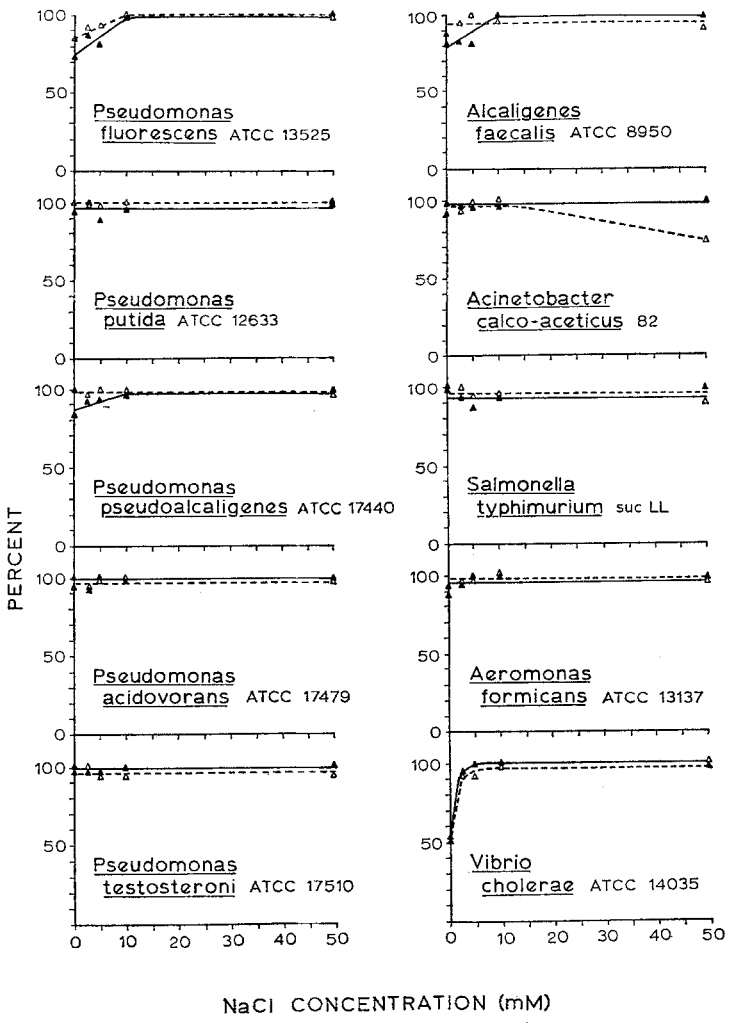

Fig. 5. Effect of NaCl on the growth of terrestrial bacteria in terrestrial medium. ▲——▲ Yield; ▵- - - -▵ growth rate. All values have been normalized by making the maximal growth rate (doublings per hour during exponential phase) and yield equal 100%

albensis had a growth rate identical to that obtained at 10, 30, and 50 mM NaCl and reached a yield which was 70% of that found at 50 mM NaCl. Furthermore, luminescence was observed in each subculture in the absence of added NaCl, a finding in disagreement with Hendrie *et al.* (1970) who stated that 85—510 mM NaCl was required for the luminescence of this strain.

Discussion

The effect of NaCl on the yields of a limited number of marine strains has been previously studied by MacLeod (1965, 1968). The present work extends this study by quantitating the effect of NaCl on the growth rates and yields of 31 gram-negative, heterotrophic, marine isolates which included polarly flagellated aerobes having moles % GC contents in their DNAs of 30.6 to 63.8 (*Alteromonas*, *Pseudomonas*, and groups undesignated with respect to genus), peritrichously flagellated aerobes having GC contents of 57.7 to 67.9 moles % (*Alcaligenes*), facultative anaerobes having moles % GC contents of 39.0—47.2 (*Photobacterium*, *Beneckea*), and a host-independent, marine bdellovibrio. All of these strains were tested for growth at different NaCl concentrations in a medium used for the cultivation of terrestrial bacteria (TM) as well as in a medium designed for use with marine bacteria (MM). The latter medium contained Mg^{++} and Ca^{++} at approximately the concentrations found in sea water (Lyman and Fleming, 1940). The NaCl concentration necessary for optimal growth rate and yield in MM varied considerably (ranging from 70—300 mM) for representative strains of different species and groups. Experiments on a limited number of strains of the same species suggested that the response of growth rate and yield to NaCl may be similar for strains of a single species. Our results with strain 121 of *Alteromonas haloplanktis* were similar to those obtained by MacLeod (1968) with strain 214 (B-16) of this species; both strains required 200—300 mM NaCl for optimal growth in MM.

A majority of the marine strains grew in both MM and TM. In general, the requirement for NaCl for maximal growth rate and yield was usually greater in TM than in MM, and, furthermore, many strains had a generation time which was considerably shorter in the latter than in the former medium. For representative strains of groups B-1, I-2, H-1, and I-1, all of which grew well in MM, TM was an inadequate medium, regardless of the concentration of NaCl present. Since MM differed from TM in having higher levels of Mg^{++} and Ca^{++} (present at approximately sea water concentrations), these results indicate that, in many cases, a medium used for the cultivation of terrestrial organisms cannot be modified to support the optimal growth of marine bacteria by the addition of NaCl alone. This conclusion is in agreement with Watson (1965), Watson and Waterbury (1971), and Williams and Rittenberg (1957) who have noted that a terrestrial medium to which NaCl has been added is not adequate for the cultivation of marine, nitrifying bacteria or marine spirilla. The NaCl concentration necessary for optimal growth of marine strains (70—300 mM) was considerably lower than the Na^+ concentration present in sea water (450—480 mM). Some of the strains studied were inhibited by sea water concentrations of NaCl, a finding previously noted by other

workers who have generally used sea water at 50—75% of its full strength (MacLeod and Onofrey, 1956; ZoBell, 1941).

The inability of strains representative of groups B-1, I-2, H-1, and I-1 to grow in TM distinguishes these organisms from the remaining marine isolates studied. This distinction is supported by a recent study of the allosteric control of aspartokinase activity in non-fermentative, marine eubacteria (Baumann and Baumann, 1974). Representative strains of groups B-1 and I-2 as well as groups H-1 and I-1 were found to have a similar pattern of control which was distinct from the remaining aerobic species and groups characterized by Baumann *et al.* (1972).

Gram-negative terrestrial strains of the genera *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Salmonella*, *Aeromonas*, and *Vibrio* were all found to grow in a medium containing 0.068 mM Na^+. In most of the strains, NaCl did not affect growth in the range of concentrations tested while in some strains a significant stimulation was observed. The highest increase in growth rate and yield was observed with four strains of *Vibrio cholerae* which grew optimally at 2.5—5.0 mM NaCl. In media containing no added NaCl these strains had a growth rate and yield which was 40—50% of the maximum. Although we did not test host-independent, terrestrial bdellovibrios for growth in TM, Ishiguro (1973) has found that a host-independent strain of terrestrial origin was able to grow in a medium similar to the one used in this study but containing no added NaCl. These results suggest that terrestrial bdellovibrios are similar to the other non-marine strains studied in that they do not require NaCl or have a requirement considerably lower than that found in marine isolates.

The results of this study support our previous suggestion that the gram-negative bacterial flora of marine origin is distinct from the gram-negative terrestrial flora. The existence of species unique to the oceans has been previously suggested by Stanier (1941). Our extensive characterization of a large number of heterotrophic marine strains has indicated that, phenotypically, these organisms form species and groups which are distinct from characterized organisms of terrestrial origin. The present work further extends this conclusion by showing that strains representative of marine species and groups have ionic requirements which are a reflection of their marine habitat. Comparable ionic requirements were not found in 14 gram-negative, terrestrial strains which had general physiological and morphological similarities to the marine isolates. The requirement for sodium found in all the marine strains characterized in our laboratory may also be found in bacteria which live in environments in which this ion is present in relatively high constant concentrations. Such an environment is provided by the rumen, which appears to harbor a number of bacteria having a relatively high requirement for this ion (Bryant *et al.*, 1959; Hudson and Caldwell, 1972; Caldwell *et al.*, 1973).

It should be noted that growth of bacteria on common laboratory media does not rule out a sodium requirement since most commercial preparations contain sufficient amounts of added or contaminating Na^+ to support appreciable growth of some marine bacteria. Our results indicate that 1% yeast extract and 1% peptone contain 7.5 and 7.2 mM Na^+, respectively. MacLeod and Onofrey (1963) found that 1% trypticase contains 28 mM and 2% agar contains 9.2 mM contaminating sodium ion. The qualitative determination of a sodium requirement in bacteria of marine origin is best performed in the 2 defined media proposed by MacLeod (1968). One of these media contains artificial sea water; the other differs by the substitution of an equimolar amount of potassium for sodium. Furthermore, since some common gram-negative bacteria of terrestrial origin require sodium for the utilization of only a single carbon compound, the usefulness of the qualitative test can be extended by employing a mixture of several carbon sources (Baumann *et al.*, 1972). Finally, our results indicate that the relatively simple artificial sea water of MacLeod (1968) containing 200—300 mM NaCl is adequate for the cultivation of a considerable number of diverse marine bacteria; however, they do not preclude the possibility that some marine organisms may require a more complex artificial sea water containing additional ionic constituents.

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Dr. Paul Baumann
Department of Bacteriology
University of California
Davis, California 95616, USA