BACTERIOLOGICAL REVIEWS, Mar., 1965 Vol. 29, No. 1

Printed in U.S.A.

The Question of the Existence of Specific Marine Bacteria[1]

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INTRODUCTION

Much information has accumulated over the past 60 years on the nutrition and metabolism of bacteria from nonmarine sources. In contrast, little comparable information is available regarding bacteria from the sea. This may be due, at least in part, to the fact that there has been considerable doubt as to whether or not there actually are specific marine bacteria. Representatives of most of the well-defined bacterial genera found growing on land and in freshwater have been isolated from seawater and marine muds. If no differences exist between bacteria in the sea and their counterparts on land except superficial ones readily lost by training, there would be little purpose in studying the nutrition and metabolism of the same genera of bacteria in more than one habitat. In fact, it has been stated that the central problem of marine microbiology is the question of the existence of specific marine bacteria, and, until this problem is settled, work on marine bacteria, apart from studies on gross transformations of matter, would have very little point (76).

Most of the experiments undertaken in attempting to settle the question have dealt with the temperature range and halophilic nature of bacteria from the sea. Marine bacteria were found to be generally more psychrophilic in character than terrestrial species and to prefer seawater or 3% NaCl to freshwater in the medium for growth. Evidence was presented, however, to indicate that these physiological properties were unstable. Baars (2), for instance, reported success in interconverting, by training procedures, three varieties of sulfate-reducing bacteria which, on the basis of temperature range, salt range, and habitat, had been regarded as separate species. ZoBell and Rittenberg (92) found that chitinoclastic bacteria from the sea, after prolonged laboratory cultivation or acclimatization procedures, developed the ability to grow in freshwater media. This change was accompanied by a widening of the temperature range for growth.

Reports on the stability of the halophilic character of marine bacteria have been particularly

[1] Issued as Macdonald College Journal Series Number 520.

confusing. Korinek (36) stated that after cultivation for 1 year on laboratory media original differences in salinity requirements between freshwater and marine bacteria were not eliminated. Stanier (76) reported failure to train marine agar-digesting bacteria to grow at appreciably lowered seawater or salt concentrations. Littlewood and Postgate (40), studying strains of *Desulfovibrio desulfuricans* of both freshwater and saltwater origin, found a complete gradation of behavior towards NaCl within the genus *Desulfovibrio*, ranging from very salt-sensitive to salt-requiring types. The strain requiring NaCl could not be adapted to grow in media lacking added NaCl. ZoBell and Michener (86), however, observed that 9 of 12 cultures requiring seawater in the medium on initial isolation grew in the same medium prepared with freshwater after the cultures had been held 5 months without transfer. Paradoxically, attempts to train the original cultures to grow at lower seawater concentrations met with only limited success. ZoBell reported subsequently that 56 of 60 species of marine bacteria had developed a capacity to grow in freshwater media (89). Observations such as these led ZoBell and Upham (88) to define marine bacteria as being bacteria from the sea which on initial isolation required seawater in the medium for growth.

Reports of the growth of marine bacteria in media prepared without seawater or NaCl were all based on observations made with complex laboratory media such as nutrient broth, fish broth, or Trypticase. This type of medium could be expected to be contaminated with inorganic ions, a factor which might conceivably have a bearing on the conflicting reports on the stability and specificity of the salt requirements of marine bacteria. The importance of inorganic contaminants for the growth of marine bacteria was first clearly demonstrated by Richter (70) in 1928, but his observations were generally ignored. By using low concentrations of peptone and taking very special precautions to avoid the introduction of inorganic contaminants, he was able to show that a marine luminous bacterium had a specific requirement for Na^+ for growth and luminescence.

Recently, studies of pure cultures of marine bacteria growing in chemically defined media have been conducted for the purpose of critically evaluating the role of the various components of the media in the growth and metabolism of the cells. From the observations made, as well as from investigations with washed-cell suspensions, cell-free extracts, and particulate components of the organisms, new insight has been gained into the relation of marine bacterial cells to their environment. These studies and the bearing of the findings on the question of the existence of specific marine bacteria are the subject of the present review.

ORGANIC REQUIREMENTS

The highest plate counts on seawater and marine materials are obtained when the plating medium contains a complex carbon and nitrogen source such as peptone. Those bacteria of marine origin which grow on such a medium prepared with seawater but not on the same medium prepared with freshwater on initial isolation have been defined as marine bacteria and their characteristics, unless otherwise indicated, are the subject of the present review. The bacteria isolated from such media are heterotrophic, 95% are gram-negative rod forms, and most are motile (89). The first attempts to replace the complex carbon and nitrogen source with chemically defined components in media for the growth of these organisms were made with marine luminous bacteria. Mudrak (58) showed that 10 strains of luminous bacteria isolated from various marine fish grew and luminesced well in nutrient solutions in which peptone was replaced by asparagine or aspartic acid. Bukatsch (12) found that several amino acids, such as glutamic acid, serine, alanine, and leucine, could replace peptone in a medium containing glycerol for the growth of some marine luminous bacteria isolated from herring. Ostroff and Henry (60) studied the capacity of 15 aerobic bacteria of marine origin to utilize various classes of nitrogen-containing compounds as sources of carbon and nitrogen in a simple medium containing 3% NaCl. The different bacteria grew luxuriantly on amino acids which, as a class of compounds, were the best sole source of nitrogen and carbon. Alanine, aspartic acid, and glutamic acid, tested separately, permitted growth of the largest numbers of different organisms. Doudoroff (22) found that four species of *Photobacterium* were able to develop in inorganic media with simple organic compounds as sole carbon source and NH_4Cl as a nitrogen source. In contrast, most strains of *P. phosphoreum* did not develop readily in the basal medium with a single carbon source but required the further addition of methionine. MacLeod et al. (43) investigated the organic nutritional requirements of 33 bacteria of marine origin; 19 were found to have relatively simple nutritional requirements, in that any one of several organic compounds could act as a source of carbon and energy with an inorganic ammonium salt present as a source of nitrogen. More organisms could use succinate as sole carbon source than could utilize glucose. The one carbon and energy source ac-

ceptable to all the organisms tested was a complex mixture of 18 amino acids. Seven of the organisms grew only in the presence of the amino acids. For the latter bacteria, the complex mixture could be replaced by glutamic acid, preferably in combination with alanine and aspartic acid. Burkholder and Bornside (13) showed that a number of marine isolates from the coast of Georgia, which were able to decompose marsh grass, did not possess specific requirements for single amino acids but grew better on multiple mixtures. Among the combinations of pure amino acids studied, a mixture of alanine, aspartic acid, and glutamic acid was reported to yield very good growth.

MacLeod et al. (43) found that several marine bacteria required the addition of vitamins to the medium for growth. Requirements for biotin, for biotin and thiamine, and for biotin, thiamine, and niacin were demonstrated in the case of three organisms. Surface-active agents stimulated the growth of two others. One organism, a *Flavobacterium*, required six amino acids, biotin, thiamine, a combination of three nucleosides, and a surface-active agent in the medium to promote appreciable growth in the absence of yeast extract (47).

Burkholder (14) reported on the general growth requirements of 1,748 aerobic heterotrophic bacteria isolated from marine muds. He was able to grow 75% of these on media of known chemical composition. Biotin and thiamine were the vitamins most frequently required for growth. Cobalamin and nicotinic acid stood next, and pantothenate and riboflavine requirements occurred infrequently.

Studies so far indicate that the marine bacteria which grow on complex media possess a wide range of organic nutritional requirements, from the relatively simple to the very complex. The plankton in seawater and its residues in marine mud could be expected to serve as a source of the nutrients required by these organisms. Although these bacteria appear to have a characteristic preference for amino acids as a carbon, nitrogen, and energy source, there is nothing that could be considered unique about their organic nutritional requirements.

INORGANIC REQUIREMENTS

The need for seawater or NaCl in the medium for growth has long been considered to reflect a requirement of marine bacteria for a medium in which salts maintained a suitable osmotic pressure. This conclusion stemmed from observations that marine luminous bacteria lysed when suspended in seawater too greatly diluted with distilled water (31, 32, 33). The first indication that there might be a specific function for the ions of seawater in the growth of marine bacteria was provided by Richter (70), who showed that a marine luminous bacterium had a specific requirement for Na^+. This report was confirmed, and the observation was extended to 10 additional strains of marine luminous bacteria by Mudrak (58), who used a chemically defined medium for the growth of his organisms. Bukatsch (12), using a defined medium, showed that luminous bacteria of marine origin also required K^+. Dianova and Voroshilova (21), employing a fish broth medium, found that Na^+ salts were required for the growth of a number of marine isolates and could not be replaced by equimolar concentrations of K^+ salts. MacLeod and Onofrey (44) found that six marine isolates grew relatively poorly when either natural or artificial seawater was the diluent in a chemically defined medium unless a supplement of an iron salt was added. In addition, both the rate and extent of growth were increased when half-strength, rather than full-strength, seawater was used. When both natural and artificial seawater were supplemented with iron and tested at half strength, there was no significant difference in the capacity of the two diluents to promote the growth of the organisms. When the need for the various ions in artificial seawater was examined, all of the organisms tested could be shown to require Na^+, K^+, Mg^{++}, $PO_4^{\equiv}$ and $SO_4^{=}$ for growth. Several of the organisms also required Ca^{++} and some Cl^-.

Requirement for Na^+

Specificity of the requirement. Since the possession of a requirement for Na^+ for growth distinguishes marine bacteria from most nonmarine species, the characteristics of the Na^+ requirement are of special interest.

When the quantitative requirements of three marine bacteria for Na^+ were determined, MacLeod and Onofrey (46) found that the maximal rate and extent of growth was achieved with 0.2 to 0.3 M Na^+, which is about one-half of the Na^+ concentration in seawater. Below this level, the rate and extent of growth were roughly proportional to the amount of Na^+ added. After a sufficiently long incubation period, growth occurred at almost one-tenth of the optimal concentration of Na^+, but never in the absence of the ion. Li^+, Rb^+, and Cs^+ showed no capacity to replace Na^+ for the growth of the organisms. K^+ exhibited a very slight sparing action at suboptimal concentrations of Na^+, an effect which disappeared on longer incubation. Sucrose had about the same limited capacity to spare the Na^+ requirement. These findings indicated that the requirement for Na^+ of the organisms examined was highly specific and that Na salts had little

if any osmotic function. Two of the organisms examined in this study have been recognized more recently to be pseudomonads, and the third was a *Cytophaga* species (52).

Payne (61) studied the Na^+ requirement of a glucuronate-oxidizing marine pseudomonad and also concluded that the effects of salts could not be explained by their osmotic action. Pratt and Austin (66), on the other hand, found that a number of salts and sucrose could greatly reduce but not eliminate the requirement of a marine *Vibrio* for Na^+. They concluded, in the case of this organism and three others examined, that a considerable proportion of the salt requirement was needed to satisfy the osmotic demands of the organisms. In an extension of these studies, Pratt (67) reported that, with seawater samples plated on a Trypticase medium containing a low concentration of added NaCl, increases in counts were obtained when the medium was supplemented with either KCl or sucrose. The counts were not so high as those obtained when the medium was made equiosmolar with respect to NaCl. He concluded that approximately half the bacteria in the samples would grow in media in which a substantial replacement of NaCl by sucrose or KCl had been made. It would thus appear that different marine bacteria differ in the extent to which nonspecific solutes can replace Na^+ for growth. In all cases examined in detail, however, it has been shown that bacteria of marine origin requiring seawater in the medium for growth have an irreplaceable minimal requirement for Na^+. Tyler et al. (80) studied 96 isolates of marine bacteria from Atlantic coastal waters off Florida and found all to require Na^+.

Most marine bacteria which have been examined have Na^+ requirements which are readily detectable, because the amounts needed for optimal growth are 0.2 to 0.3 M. Such organisms require the addition of Na salts or seawater even to the complex laboratory media commonly used for their isolation, though such media are usually contaminated with appreciable amounts of Na^+. Two organisms of marine origin were isolated, however, which grew optimally in complex media prepared with freshwater. Such organisms would not have been classified as marine bacteria according to earlier criteria (88). When grown on chemically defined media, however, their requirements for Na^+ became apparent (43). Quantitative estimations of their requirements revealed that one needed 0.02 M Na^+ and the other 0.005 M Na^+ for optimal growth (MacLeod and Onofrey, *unpublished data*). By comparison, the nutrient broth-yeast extract isolation medium prepared with distilled water contained 0.03 M Na^+, an amount clearly sufficient to permit optimal growth of both organisms.

Stability of the requirement. It was of interest to determine whether the requirement of marine bacteria for Na^+ is as readily lost as the requirement for seawater had been reported to be. By plating heavy suspensions of marine bacteria on Trypticase medium prepared without added Na^+, Pratt and Waddell (63) obtained a few colonies which they concluded were mutants of marine bacteria no longer requiring Na^+ for growth. MacLeod and Onofrey (53) trained a marine pseudomonad to grow on Trypticase medium prepared without added Na^+ salts by streaking cultures serially onto the surface of plates of the medium containing progressively lower concentrations of Na^+. A flame photometric analysis of the Trypticase medium without added salts revealed a concentration of 0.028 M Na^+ present as a contaminant. When the adapted culture was tested in a chemically defined medium containing less than 6.5×10^{-5} M Na^+, the organism was found still to require Na^+ for growth. The adapted culture grew only a little more quickly and at a slightly lower Na^+ concentration in the chemically defined medium than the parent culture. All attempts to train the organism to grow in the chemically defined medium in the absence of added Na^+ failed. This organism, which had been trained to grow in a complex medium without added Na^+ salts had apparently developed a capacity to grow well at the concentrations of Na^+ present as a contaminant in the complex medium, so long as other components of the complex medium were present. The possible significance of this finding in relation to the reported ability of some marine bacterial cultures to lose their requirement for seawater remains to be established.

When a marine pseudomonad was exposed to ultraviolet irradiation, a limited number of what appeared to be mutants were obtained which grew in the chemically defined medium in the absence of added Na^+ (53). The extent and rate of growth of the mutants was still enhanced by added Na^+, but this response could be eliminated by training. The difficulty experienced in getting any appreciable number of mutants lacking a Na^+ requirement by irradiation of a Na^+-dependent culture is a further indication of the stability of the Na^+ requirement of these organisms.

Uniqueness of the requirement. The evidence which has accumulated suggests that bacteria from the sea which require seawater in the medium for growth on isolation possess a stable, highly specific, and in most cases readily detectable requirement for Na^+ for growth. To

what extent is this a characteristic unique for marine bacteria? Halophilic bacteria, including representatives of the extreme halophiles, have been isolated from freshwater sources and soil. Both extreme and moderate halophiles have been reported to have specific requirements for Na^+ [*see* Larsen (39) for a review]. Among nonhalophilic species, two strains of *Rhodopseudomonas spheroides* and one strain of *R. palustris* were found to require Na^+ when grown in a chemically defined medium (75). The original source of these isolates was unknown. A strain of *Bacteroides succinogenes*, a cellulolytic organism isolated from the rumen of a steer, also has been shown to require Na^+ for growth (10). Goldman and co-workers made the interesting observation that a number of strains of lactic acid bacteria isolated from meat-curing brines developed a requirement for NaCl at elevated temperatures (28). Tests indicated that neither the Na^+ nor the Cl^- could be replaced by other ions. These are the only well-documented cases so far reported of bacteria from nonmarine sources requiring Na^+ specifically for growth. Sakazaki et al. (71), however, reported that they have confirmed an observation made by Nakagawa (cited as a personal communication) that various halophilic organisms can be found in the feces of guinea pigs, rats, and monkeys. These organisms required 3% salt for growth. Although a requirement for Na^+ has not been established in this case, it is evident that a specific need for Na^+ is not a characteristic unique for bacteria of marine origin and may well prove to be more widespread than was previously imagined.

Among those organisms needing Na^+ for growth, there is a wide range in quantitative requirements. In the case of extreme halophiles, growth ceased when the Na^+ concentration fell below 1.5 M, even in the presence of large amounts of K^+ or Mg^{++}, and for maximal growth under these circumstances 2.5 M Na^+ was required (5). The moderate halophile *Vibrio costicolus* had a nonspecific requirement for about 0.4 M salt in the medium but a specific requirement for only 0.017 M Na^+ (18). The strains of *Rhodopseudomonas* studied by Sistrom (75) required a maximum of 0.002 M Na^+ for growth. The marine bacteria so far examined have been found to have optimal requirements for Na^+ ranging from 0.005 to 0.2 M, depending on the species.

Function of Na^+. Washed-cell suspensions of two marine pseudomonads were shown to require Na^+ as well as K^+ for the oxidation of exogenous substrates (79, 48, 62). In the case of these organisms, neither related ions nor sucrose showed any significant capacity to reduce the requirement for Na^+ for oxidation. In this respect, the responses to the ions for substrate oxidation were similar to those for growth. When one of the organisms was examined in more detail, the amounts of Na^+ required for oxidation were found to vary, depending on the substrate being oxidized (48). To obtain maximal rate of oxidation of acetate, butyrate, propionate, or an oxidizable sugar, 0.05 M Na^+ was required; for malate, citrate, and succinate, 0.15 to 0.20 M Na^+ was necessary. All the enzymes of the tricarboxylic cycle were found to be present in cell-free extracts of the organism. When each of the enzymes was tested for its response to inorganic ions, the acetate-activating enzyme and malic dehydrogenase were found to require K^+, aconitase and isocitric dehydrogenase required media of appropriate ionic strength (0.3 to 0.4 μ) for optimal activity, and the remainder functioned better in the absence of added salts than in their presence. None of the enzymes, however, could be shown to require Na^+ specifically (48, 49).

Washed cells of a marine *Vibrio* species were found to require both Na^+ and K^+ for the production of indole from tryptophan (64). In the case of this organism, however, the presence of sucrose in the suspending medium reduced the Na^+ requirement for indole production from 0.3 to 0.05 M. A similar sparing action of sucrose on the Na^+ requirement has been observed with this organism during growth (66). Cell-free extracts of the *Vibrio* required K^+ and pyridoxal phosphate for indole production. Added NaCl was not required, and concentrations of NaCl giving optimal activity with intact cells partially inhibited the activity of cell-free extracts.

Payne noted that induction and activity of enzymes for catabolizing glucuronate in the marine isolate *Pseudomonas natriegens* were specifically affected by the presence of Na^+ and K^+. The role of K^+ appeared to be restricted to influencing the activity and not the induction of enzymes. The requirement for Na^+, however, seemed to be coupled to the induction of a mechanism for the uptake of glucuronate (61, 62). In subsequent experiments, the induction of resting cells of the same organisms and other marine isolates to the oxidation of L-arabinose, mannitol, and lactose was found to be dependent on the presence of Na^+ (69).

A role for Na^+ in the induction of penetration mechanisms or in the formation of adaptive enzymes would fail to account for the requirement for Na^+ observed when compounds were oxidized by pathways employing constitutive permeases and enzymes. Under these circumstances, since whole cells required Na^+ for the metabolism of

substrates whereas intracellular enzymes appeared not to require the ion, it seemed likely that Na^+ might be involved in the transport of substrates into the cell. To test this possibility, it was necessary to dissociate the uptake of substrates from their subsequent metabolism. This was accomplished by using nonmetabolizable analogues of metabolizable substrates. Drapeau and MacLeod (23) found that, when washed cells of a marine pseudomonad were incubated with C^{14}-α-aminoisobutyric acid, this analogue of the naturally occurring amino acids accumulated inside the cells but could not be metabolized. The uptake required the presence of Na^+ in the suspending medium. Since uptake took place without a lag period from an incubation mixture containing chloramphenicol, the possibility that the accumulation was due to the preliminary induction of a penetration mechanism was rendered very unlikely. K^+, Rb^+, NH_4^+, Li^+, and sucrose could not substitute for Na^+ in the transport process. Sulfate and chloride salts providing the same level of Na^+ were equally effective. The uptake process was an active one, because the substrate was concentrated in the cells to a level some 3,000 times that in the medium. The uptake was stimulated by the presence of an oxidizable substrate (in these experiments, galactose). Since galactose required less Na^+ for its maximal rate of oxidation than was needed for the optimal rate of uptake of the amino acid analogue, there was clearly a role for Na^+ in the uptake process which was separate from any other possible role of Na^+ in oxidative metabolism. Because D-fucose, a nonmetabolizable analogue of galactose, also required Na^+ for uptake, it seemed likely that the requirement for Na^+ for galactose oxidation also represented a requirement for transport. The uptake of α-aminoisobutyric acid by cells of the marine luminous bacterium *Achromobacter* (*Photobacterium*) *fischeri* has also been shown to be a Na^+-dependent process (Drapeau and MacLeod, *unpublished data*). These results support the conclusion that the primary function of Na^+ in marine bacteria may be to permit the transport of substrates into the cell. Previously observed differences in the quantitative requirements for Na^+ for the oxidation of various substrates by cells of a marine bacterium (48) can now be accounted for if one assumes a number of different permeases in the cell membrane with quantitatively different requirements for Na^+ for activation. Whether or not there are Na^+-dependent transport mechanisms in Na^+-requiring bacteria of nonmarine origin has yet to be determined.

Response to Halides

Of six marine bacteria examined, three were found to have an absolute requirement for halide ions for growth, and three reached maximal growth more quickly if halide was present in the medium (45). Chloride and bromide could be used interchangeably on a mole for mole basis in these experiments. Iodide was toxic. The amounts of halide required and the effects of the anion on rate and extent of growth corresponded closely to the response to Na^+, suggesting that the function of the two ions might be closely related in the metabolism of those organisms requiring both ions.

Some moderate and extreme halophiles have specific requirements for chloride for growth, whereas others do not [*see* Larsen (39) for a review]. In all cases but one so far reported, halide requirements for growth among bacteria have been detected only in bacteria which also need Na^+ specifically, though organisms requiring Na^+ do not always need halide. The exception is a strain of *D. desulfuricans* which failed to grow without the addition of NaCl to the medium. The Na^+ but not the Cl^- could be replaced by other ions (40).

Requirement for Mg^{++}

When grown in a chemically defined medium, marine bacteria have been found to require 4 to 8 mM Mg^{++} for maximal rate and extent of growth (45). This requirement is high compared with that of most terrestrial species examined. A level of 0.02 mM Mg^{++} was established as the requirement of a strain of *Escherichia coli* (85) and 0.08 mM was needed by *Bacillus subtilis* (26). Wiebe and Liston (83), noting the high Mg^{++} requirement of classical marine bacterial types, suggested that this might be a useful criterion of the marine origin of a bacterium. In the case of the marine bacteria examined by MacLeod and Onofrey (45), however, a marked interaction between Mg^{++} and Ca^{++} was noted. For one organism, the presence of 2.5 mM Ca^{++} in the medium reduced the requirement for Mg^{++} from 8 mM to 0.04 mM but did not eliminate the need for the ion. Higher levels of Ca^{++}, on the other hand, increased the requirement for Mg^{++}. For other organisms, both Mg^{++} and Ca^{++} were required for growth and, in these, the quantitative requirements for one of the ions was much affected by the level of the other in the medium. Both Mg^{++} and Ca^{++}, therefore, appear to play an important role in the nutrition of marine bacteria, and the requirements for these two ions taken together are somewhat higher than those of most terrestrial species. The requirements of marine bacteria for divalent ions are low, however, compared with the levels required by the extreme halophiles. For the latter organisms, 100 to 500 mM concentrations of Mg^{++} are necessary for optimal growth and for the maintenance of

normal morphology in media containing all the other ions necessary for growth at their optimal concentrations (5).

Salt Tolerance

It is commonly assumed that marine bacteria, since they live in the sea, must be salt-tolerant organisms. Seawater, however, contains only 0.45 M Na^+, 0.05 M Mg^{++}, 0.01 M K^+, and 0.01 M Ca^{++}, plus traces of other ions. The Na^+ level, expressed as NaCl, is about 2.6%. Three marine bacteria investigated by MacLeod and Onofrey (46) were inhibited by the presence of 0.8 M Na (4.7% NaCl) in the medium. Of 15 marine bacteria examined by Tyler et al. (80), all grew at 0.8 M (4.7%) NaCl, 9 grew at 1.4 M (8.2%) NaCl, and none grew at 2.6 M (15.2%) NaCl. In contrast, many terrestrial species, among them organisms not classed as halophiles, can tolerate much higher concentrations of salt than the marine bacteria studied. Larsen (39) stated that, among bacteria commonly found to be agents of food spoilage, aerobic sporeformers grow at 15 to 20% NaCl and many micrococci tolerate 25% NaCl. Gram-negative rods of terrestrial origin are generally completely inhibited by NaCl concentrations between 5 and 10%, and thus have a sensitivity to salt similar to that of the marine bacteria examined.

Lytic Susceptibility

Characteristics of the lytic phenomenon. Harvey (31) observed in 1915 that marine luminous bacteria failed to luminesce when the seawater in which they were suspended was too greatly diluted with distilled water. He ascribed the effect to cytolysis through lowered osmotic pressure, because light production was maintained when seawater was replaced by a 1 M sucrose solution. Hill (32) concluded that luminous bacteria are cytolyzed by water, hypotonic nonpenetrating solutions, and penetrating solutions of all concentrations. A penetrating solution in Hill's study was one which failed to prevent lysis of cells suspended in it. In a study of 96 isolates of marine bacteria (all nonluminous gram-negative rod forms), Tyler et al. (80) observed that in the majority of cases suspensions of cells of the organisms were susceptible to a loss of optical density in distilled water. MacLeod and Matula (52) found that five marine bacteria differed considerably in lytic susceptibility. Two lysed immediately and completely when suspended in less than 0.15 M NaCl, but suspensions of the other three still contained many whole cells at 0.025 M NaCl.

Pratt and Riley (65) and MacLeod and Matula (52) noted differences in the capacities of different salts to prevent lysis of marine bacteria. For a number of different isolates NaCl and LiCl were found to be more effective than KCl or NH_4Cl in preventing lysis. The same salts had the same relative capacity to prevent lysis in the case of the moderate halophile *Vibrio costicolus* (19) and the extreme halophile *Halobacterium cutirubrum* (1), suggesting that the mechanism of lysis may be basically the same in all of the organisms examined.

Divalent cations were found to be much more effective than monovalent cations in preventing lysis of marine bacteria (51). The order of effectiveness of the divalent cations appeared to be similar to that of their capacity to form chelate complexes. The Mg^{++} and Ca^{++} concentrations in seawater would have been sufficient to prevent the lysis of all but one of the marine bacteria examined, without the assistance of Na^+ salts.

The nature of the anion was found to be important in preventing lysis of marine bacteria, particularly on long incubation of suspensions of the cells (52). For four of five organisms examined, sulfate salts stabilized the cell suspensions better than did chlorides. For the fifth organism, the reverse was true.

As little as 5×10^{-4} M spermine was found to suppress lysis of the marine luminous bacterium *Achromobacter fischeri* (41).

Mechanism of lysis. The wide variation in the concentration of the different solutes required to prevent lysis made it seem extremely unlikely that all the solutes exerted their effects through osmotic action. Proof that NaCl does not prevent lysis in this way in the case of one marine pseudomonad was obtained by measuring the intracellular Na^+ and Cl^- concentrations at various levels of extracellular NaCl (78). At all levels of Na^+ in the medium, the intracellular and extracellular Na^+ concentrations within the limits of experimental error were the same. Intracellular and extracellular Cl^- concentrations were the same at the one level of Cl^- examined. Since, so far as NaCl was concerned, no gradient was maintained between the inside and outside of the cell, NaCl could not prevent lysis of the cells through osmotic action.

Brown (6, 7) prepared cell walls of a marine pseudomonad by mechanical disintegration of the cells followed by washing with distilled water. Suspensions of the cell walls, when incubated in a dilute phosphate buffer (0.05 M), showed a decrease in absorbancy with time. This decrease was prevented by increasing the buffer concentration, by heating the cell walls, or by the addition of spermine. When cell walls were incubated under conditions permitting a decrease in absorbancy of their suspensions, a dialyzable fraction and a nondialyzable fraction were released. An acid hydrolysate of the nondialyzable fraction was shown to contain hexosamine, muramic acid,

and the normal amino acids of protein hydrolysates. Both diaminopimelic acid and glucose, constituents of the cell-wall residue, were absent from the nondialyzable fraction. The dialyzable fraction contained a number of peptides. The latter observation suggested to Brown that the breakdown of the crude cell wall is caused by a lytic enzyme in the cell wall, and not merely by spontaneous disintegration under appropriate physicochemical conditions. Comparison of the effect of cations on the cell-wall autolytic system and on tryptic digestion of the cell envelope suggested to Brown (8) that the simplest and most probable explanation of the effects of ionic strength and particularly di- and multivalent cations was that they operate through their influence on the conformation of membrane proteins. Proteolytic autolysis was considered to be a direct consequence of such changes.

Buckmire and MacLeod (11) did not favor this hypothesis, because the lysis of whole cells is such a rapid process that it seemed unlikely that it could be due to the action of an enzyme. Cell envelopes of a marine pseudomonad were prepared by mechanical disintegration of the cells in 0.5 M NaCl, a concentration of salt able to prevent lysis. The envelopes were washed free from cytoplasmic material with 0.5 M NaCl. This was a departure from the procedure of Brown, who washed his cell envelopes in distilled water. It was felt that this might well lead to the loss of components important in the maintenance of cell-envelope structure. When a suspension of the cell envelopes in 0.5 M NaCl was added to distilled water, a soluble nondialyzable material was found to be present in the supernatant solution. Both the nondialyzable fraction and the cell-envelope residue after acid hydrolysis contained glucosamine, muramic acid, 15 amino acids (including diaminopimelic acid), and four unidentified ninhydrin-positive compounds. It appeared from visual inspection of the paper chromatograms that not only were the same compounds present in both fractions but that they were present in the same relative proportions. When cell envelopes suspended in 0.5 M NaCl were heated at 100 C for 15 min, they still released the nondialyzable hexosamine-containing fraction on suspension in distilled water. When the suspension of walls in 0.5 M NaCl was autoclaved at 121 C for 10 min, a considerable release of hexosamine-containing material occurred. This could be largely prevented, however, by raising the NaCl concentration to 5 M. The effect of heat and salt concentration on the release of the hexosamine-containing fraction is exactly analogous to the effects of heat and salts on the denaturation of a polyanion, and is explainable in terms of polyelectrolyte theory (37).

The finding that a fraction is released from the cell envelopes into distilled water, which has apparently the same composition as the residual cell envelope, suggested that the cell envelope is made up of a series of units. The effect of heat and salt concentration could best be explained if one assumes that the units are held together by cross-linkages between polyanions on adjacent units. The units would be able to come close enough together to form a continuous wall only if the negative charges on the polyanions were screened by the cations of a salt. The effects of salts in maintaining the integrity of the envelopes could thus be explained satisfactorily on the basis of polyelectrolyte theory. Conditions which prevent denaturation of a polyelectrolyte maintain the structure of the envelope. This did not eliminate the possibility that an enzyme was involved, because enzymes are polyelectrolytes. An explanation of lysis based on spontaneous disintegration of the envelopes under appropriate physicochemical conditions, however, was more compatible with the observations than an explanation involving enzymes.

It would thus appear that the cell envelopes of the marine bacteria examined are maintained intact by salts in somewhat the same way as the envelopes of the more extreme halophiles. Abram and Gibbons (1) suggested that the cell walls of halobacteria are held together by hydrogen bonds, Coulomb forces, or "salt" linkages, and that in the presence of NaCl the electrostatic forces are screened so that the bonds hold the organism in a rod shape. Brown (9) concluded that the effects of salt concentration, bivalent cations, and *p*H on the disaggregation of cell envelopes of *H. halobium* are all consistent with a mechanism which operates principally through exposure on the membrane of a net negative charge.

In the case of organisms which lyse in distilled water, then, there is direct evidence through studies with isolated cell envelopes that inorganic ions are directly involved in holding the cell wall together. Evidence has been obtained which suggests that a somewhat similar situation may prevail in some terrestrial species. Repaske (68) reported that a number of gram-negative bacteria could be induced to lyse in the presence of lysozyme if the incubation mixture contained the metal-binding agent ethylenediaminetetraacetic acid (EDTA). Carson and Eagon (17) found that EDTA alone was capable of lysing a suspension of *P. aeruginosa*, producing large cell-wall fragments which could then be further digested by lysozyme. It is tempting to speculate that these fragments are units of the cell envelope which, in the intact cells, are held together by metal ion bridges. Thus, there may be more in

common in the structures of the cell envelopes of marine and terrestrial pseudomonad species than was suspected previously.

Singularity of lytic susceptibility. Among gram-negative bacteria, there is a spectrum of susceptibility to lysis ranging from organisms which require high salt concentration to prevent disruption of the cells to those which maintain their integrity in distilled water. The bacteria which are most susceptible to lysis are the extreme halophiles, the halobacteria, which lyse below 2.0 M NaCl (1). Next come the moderate halophiles, of which *V. costicolus* is an example. This organism lyses at NaCl concentrations ranging from 0.25 to 1.0 M, depending upon the salt concentration of the growth medium (19). At the lower end of the spectrum come the marine bacteria. Some species lyse when the NaCl concentration drops below 0.15 to 0.2 M. In the case of others, only part of the population lyses in distilled water (52). Organisms of terrestrial origin are ordinarily considered not to be susceptible to lysis. However, two nonmarine species, *Pasteurella tularensis* and *Neisseria perflava* are markedly affected by a lowered solute concentration, as indicated by leakage of cell material, decay of respiratory ability, and decline of viability on brief exposure to distilled water (41, 42). Furthermore, the capacity of solutes such as Mg^{++} to maintain the respiratory activity of cells of *Azotobacter* (29), an organism otherwise stable in water suspension, may represent a further ramification of a basically similar phenomenon. It is evident, therefore, that a clear-cut distinction between marine and nonmarine species of bacteria cannot be made on the basis of lytic susceptibility alone.

METABOLIC PATHWAYS IN MARINE BACTERIA

Very little information is available regarding the intermediary metabolism of marine bacteria. All the enzymes of the tricarboxylic acid cycle have been found to be present in cell-free extracts of a marine pseudomonad (48, 49). Enzymes of the glyoxylate by-pass were also detected (50). Isocitrate lyase was demonstrated in extracts of *Agarbacterium alginicum* (84). Enzymes of both the glycolytic pathway and the hexose monophosphate pathway were demonstrated to be present in extracts of glucose-grown cells of the marine pseudomonad, *P. natriegens* (24). Data from radiorespirometric experiments indicated that approximately 92% of the glucose was catabolized via the glycolytic pathway and 8% by the hexose monophosphate pathway. The factor controlling the choice of pathways in this organism has been shown to be the availability of nicotinamide adenine dinucleotide phosphate (NADP) (25). The bacterium requires NADP for the operation of the hexose monophosphate pathway but lacks pyridine nucleotide transhydrogenase and reduced NADP ($NADPH_2$) oxidase, enzymes required for the reoxidation of $NADPH_2$. That this is not a phenomenon associated exclusively with marine bacteria is evident from the fact that the hexose monophosphate pathway in bacteria from other habitats as well as in mammalian tissues is rate-limited by the supply of NADP (25).

Ochynski and Postgate (59) compared the properties of freshwater and saltwater (though not necessarily marine) strains of *Desulfovibrio desulfuricans* and found that growth in a saline environment led to the production of a mucopolymannoside not chemically related to the cell wall. An increase in the content and change in the kind of "free amino acid material" within the cell was also noted. Adaptation of a freshwater strain to a saline environment led to the acquisition of these characters and a morphological change. For an adaptive change in the reverse direction, only the last character was studied and it was not lost.

OTHER FACTORS

Relation to Temperature

Most marine bacteria examined can be described as being facultatively psychrophilic because, according to Bedford (4) and others, the majority grow at 0 C, have a temperature optimum of 20 to 25 C, and do not grow above 30 C. ZoBell and Conn (87) reported that heating samples of seawater and marine mud to 30 C for 10 min killed about 25% of the bacteria, and only 20% survived 40 C for 10 min. Psychrophilic microorganisms are, of course, very widely distributed in nature, having been isolated in appreciable numbers from air, water, soil, plants, animals, and a great variety of foods (77).

Though psychrophily is not a characteristic unique for bacteria of marine origin, its physiological basis in marine bacteria is of considerable interest. Burton and Morita (15) found that 55 to 60% of the malic dehydrogenase activity of cell-free extracts of a marine facultative psychrophile (optimal temperature for growth, 24 C; maximal, 30 C) was lost by exposure of the extract to 30 C for 15 min. The rate of denaturation of the enzyme was much greater at 35 and 40 C. Heat stability of the enzyme was found to be greater in whole cells than in cell-free extracts (56). Heating the cells or treatment with a lysing agent apparently destroyed some regulatory factor for malic dehydrogenase activity. The data indicated that this regulatory factor was cell permeability. Additional support for the conclusion that at least two factors, heat lability of

vital enzymes and membrane permeability, are involved in governing the maximal temperatures at which these organisms can grow arose from studies with an obligate psychrophile *Vibrio marinus* (optimal temperature for growth, 15 C; maximum, 20 C). Morita and Robison (57) found that temperatures from 20 to 30 C were sufficient to inactivate the metabolic systems involved in oxygen uptake, either endogenously or in the presence of glucose, in this organism. These temperatures also caused leakage of 260- to 280-mμ absorbing material. The amount of leakage was greater with increased exposure as well as increased temperature.

Evidence is accumulating that enzymes and enzyme-forming systems in other psychrophilic microorganisms are abnormally sensitive to heat (3, 30, 81).

Since more than 80% of the marine environment is perpetually colder than 5 C, factors permitting growth of marine bacteria at the lower end of their temperature range are also of concern. Of particular interest in this connection was the observation of Morita and Burton (56) that in whole cells of a marine facultative psychrophile there was a 50% decrease in malic dehydrogenase activity with each 10 C decrease in temperature down to 13.8 C. A further temperature drop to 5 C reduced the enzyme activity only 15%. Since malic dehydrogenase activity in cell-free extracts was reduced 64% over the same temperature range, the authors concluded that whole cells have some mechanism for permitting enzymes to function at low temperatures at rates which are higher than one would expect from their response to temperature in a cell-free system.

Relation to Pressure

Since the average depth of the world's oceans is more than 2 miles, and hydrostatic pressure increases roughly 1 atm for each 10 m of depth, much of the sea floor is subjected to pressures exceeding 300 atm. At the deepest points in the ocean, hydrostatic pressures approaching 1,100 atm prevail. Thousands to millions of bacteria are known to be present per gram of marine sediments (89). One might expect, therefore, that organisms able to survive and grow at the bottom of the sea would be more tolerant of pressure than terrestrial species. ZoBell and Johnson (90) compared the effects of pressure on representative species of terrestrial and marine bacteria. None of the terrestrial bacteria multiplied perceptibly at a pressure of 600 atm, and growth of most was slowed by 300 atm. Marine species from near the surface of the sea resembled the terrestrial bacteria in their sensitivity to pressure, whereas those isolated from depths, where the pressure approximated 500 atmospheres, grew readily at a pressure of 600 atm. Mixed microflora from muds of the same depth appeared to grow faster under pressure. Organisms whose growth was favored by pressure were referred to as barophiles. ZoBell and Morita (91) found bacterial populations ranging from 10^3 to 10^6 per gram of wet mud in samples taken from depths of 7,000 to 10,000 m. Counts made at a pressure of 1,000 atm (the approximate pressure prevailing at these depths) were in most cases appreciably higher than those conducted at 1 atm. The authors reported that a good many tests made on bacteria which grew at a pressure of 1,000 atm demonstrated their inability to grow in similar media incubated at 1 atm. Similarly, among the many cultures tested, none which grew at 1 atm did so when incubated at 1,000 atm.

Kriss and co-workers (38) isolated 146 strains of bacteria from deep-sea bottom deposits and from garden soil which had been subjected to high hydrostatic pressure. The organisms could be divided into two groups, those which remained viable but were unable to reproduce at 450 atm of pressure and those which were able to grow at this pressure. Only one strain was found which developed better at 450 atm of pressure than at atmospheric pressure. In general, strains growing well at 450 atm grew even better at 1 atm. These workers reported the isolation from the upper layers of the soil of cultures able to grow and reproduce at 1,500 atm of pressure.

The mechanism of action of pressure on biological systems has been extensively studied by Johnson and co-workers (34). The effects of pressure have been explained in terms of the molecular volume change accompanying a limiting reaction. The influence of pressure not only may be modified but even reversed in direction by a change in temperature. Below the normal optimal temperature, an increase in pressure may produce inhibition by opposing the molecular volume increase accompanying the limiting reaction. At temperatures above the optimum, the critical enzyme undergoes a reversible denaturation that proceeds with an even larger volume increase than the limiting reaction. At these temperatures, the net effect of pressure is to increase the rate of the reaction by reversing the denaturation of the enzyme to a greater extent than opposing the catalytic reaction. In keeping with this hypothesis, ZoBell and Johnson (90) observed that lower temperatures markedly accentuated the growth-retarding and disinfecting effects of pressure on bacterial cultures. At higher temperatures, pressure in some cases acted in the direction of opposing the unfavorable effects on growth and

viability caused by high temperature. As a further example of the effect, Morita and Haight (55) observed malic dehydrogenase activity at 101 C under hydrostatic pressure.

There are enormous technical problems associated with obtaining quantitative information on the relation of the various types of marine bacteria in deep-sea bottom deposits to pressure. It takes 8 to 18 hr to bring samples to the surface from a depth of 10,000 m (91), and no way has yet been devised to maintain samples during collection and subsequent manipulation at the pressures and temperatures prevailing in the depths. If there are bacteria at the bottom of the sea which depend upon the particular temperature-pressure combination found there to maintain the conformation of vital polyelectrolytes, many of these bacteria could well be rendered nonviable by the decrease in pressure and increase in temperature associated with bringing samples to the surface.

Taxonomic Position

It is of interest to know whether bacteria in the sea differ in a sufficient number of characteristics from bacteria in other habitats to warrant their being placed in a separate taxonomic group. Miyamoto and co-workers (54), for instance, proposed that the gram-negative polarly flagellated rod forms found widely distributed in the ocean be grouped in a new genus *Oceanomonas*. The distinctive character of this genus would be the degree of halophilism usually exhibited by marine bacteria. Sakazaki et al. (71) could not accept this proposal, since the genus would include a group of enteropathogenic marine bacteria which they concluded were vibrios. Shewan and co-workers (74) have worked out a determinative scheme for gram-negative bacteria from the marine environment which groups these organisms into the genera *Pseudomonas*, *Xanthomonas*, *Aeromonas*, *Vibrio*, *Achromobacter*, *Alcaligenes*, *Flavobacterium*, *Cytophaga* and a peritrichously flagellated group referred to as "Paracolons." Colwell and Gochnauer (20) examined 60 bacterial cultures of marine origin for approximately 100 characteristics, including Na^+ and Mg^{++} requirement, amino acid growth response, and the standard bacteriological characters. The data were coded and analyzed by electronic computer by use of the Adansonian method. Also, these data were compared by computer with other data similarly obtained for 131 named strains of the Eubacteriales and Pseudomonadales. Results of the analyses showed four groupings within the marine strains, three *Pseudomonas* and one *Vibrio* cluster. No single characteristic was exclusive to any one of the groups which were based on overall similarity. This data suggested that separate genera should not be formed to describe marine species, and is the type of result one might expect to obtain if there is a close evolutionary relationship between marine and terrestrial species. Since life is believed to have originated in the sea, it is not unlikely that the common ancestor of both marine and terrestrial bacteria was a marine bacterium. Although much remains to be done to elucidate the differences between marine and terrestrial species at the molecular level, enough information is available to suggest that a quite limited number of successive mutations could convert a marine species to a form which would not be dependent on the sea for its survival.

Capacity to Survive in Seawater

It is evident that there are bacteria in the sea which depend upon the kinds and amounts of inorganic ions in seawater for their survival. Just what proportion of the bacteria in the sea have this dependence is not yet clear. That it is probably a high proportion, at least of those bacteria able to grow on laboratory media, was made evident very early in the study of marine microbiology by the large increases in counts obtained when marine materials were plated on seawater rather than freshwater media. It is also evident that there are bacteria having some of the special characteristics of marine bacteria in other environments. Such characteristics as Na^+ dependence and lytic susceptibility will not alone stamp bacteria as being uniquely marine. Nevertheless, bacteria dependent on the inorganic composition of seawater for their survival appear to predominate in the sea, even though the possession of these inorganic requirements confers no obvious competitive advantage on the organisms.

It has long been known that seawater possesses marked bactericidal activity for a variety of terrestrial organisms. This is not a simple matter of intolerance to the concentrations of salts that are present in the sea, because seawater can be rendered nontoxic for some organisms by autoclaving and for others by adding small amounts of appropriate organic materials. Because of its relation to sewage disposal, most of the investigations have dealt with the effects of seawater on *E. coli* (16). The loss of viability which occurs when cells of this organism are suspended in either natural or artificial seawater can be prevented by adding small amounts either of cysteine or of other amino acids having the capacity to form chelate complexes with metal ions (73). Jones (35) observed that the long lag phase which occurred when *E. coli* was grown in media prepared with seawater or 2.5% NaCl could be overcome by the addition of small amounts of com-

pounds which had in common the capacity to act as chelating agents. It has been concluded by the various workers that the bactericidal action of seawater for *E. coli* is due to its content of toxic heavy metals in trace amounts. Saz et al. (72) report the presence in seawater of a non-dialyzable, heat-labile compound having rapid bactericidal activity against both penicillin-sensitive and -resistant strains of *Staphylococcus aureus*. Under the conditions of the experiments, the substance exhibited no activity against *E. coli*.

The key, then, to the distinction between marine and terrestrial bacterial species may well be the mechanism or mechanisms which confer on bacteria the capacity to survive and grow in the sea. The fact that ability to survive in the sea is linked to the possession of particular inorganic requirements is probably not fortuitous, but a direct relationship between these characteristics, if it exists, remains to be elucidated. We are forced to conclude that there are bacteria which are uniquely marine because they are able to survive and grow in the sea, and we have yet to find out why.

Relation of Organisms Isolated to Indigenous Flora

It has been known for many years [see Waksman et al. (82)] that the numbers of bacteria in seawater or marine mud able to grow on laboratory media are as many as 1,000 times smaller than the numbers observed by direct microscopic examination. This has been emphasized recently by Kriss and co-workers (38), who reported that plating on standard laboratory media detected not more than 0.1 to 1% of the total numbers of microorganisms which can be observed microscopically in seawater or mud samples. That at least some of these forms must be viable was indicated by the fact that unusual morphological types never isolated from laboratory media were capable of forming microcolonies on glass slides submerged in seawater. Furthermore, deep-sea investigations in the Black Sea, Pacific, Atlantic, and Arctic oceans showed that some of the microbial forms revealed by direct microscopic examination were widely distributed. It would thus appear that present knowledge of the properties of marine bacteria has been gained from studies on representatives of the less than 1% of bacteria in the sea able to grow under ordinary laboratory conditions. To what extent their characteristics are common also to the types of organisms yet uncultured remains to be determined. This, of course, is a problem not confined to the marine environment. Only a small percentage of the bacteria observed by microscopy in soil and fresh water ever grow on laboratory media [see Gibson (27) for a review]. We are therefore faced with the very real possibility that, in the case of many natural environments, the organisms isolated and studied may not in fact be true representatives of the indigenous population.

SUMMARY

The marine bacteria which grow on media giving the highest plate counts on seawater and marine materials are largely gram-negative rod forms most of which are motile. The majority are facultatively psychrophilic and some, particularly those from deep-sea bottom deposits, can grow at high hydrostatic pressures. Many have a preference for amino acids as sources of carbon, nitrogen, and energy, and some require vitamins and other growth factors. Metabolic pathways in these organisms appear to be similar to those in other species.

Marine bacteria have special requirements for inorganic ions, partly to supply the needs of the organisms for growth and metabolism, partly to maintain the integrity of the cells. They have a highly specific need for Na^+ for growth, which has been shown in two species to reflect the presence of a Na^+-dependent mechanism for transporting substrates into the cells. Some of the bacteria fail to grow in the absence of halide ions, and this requirement can be satisfied either by chloride or bromide. Their need for Mg^{++} or for a combination of Mg^{++} and Ca^{++} exceeds that of most terrestrial species. For some marine bacteria, the effect of salts in maintaining the integrity of the cells has been shown to be due entirely to the capacity of the salts to interact directly with the cell envelopes. For other species of marine origin salts may also have an osmotic function.

Although the marine bacteria examined have a number of characteristics in common, the only one which clearly distinguishes them from bacteria in other habitats is a capacity to survive and grow in the sea. In this respect, then, marine bacteria are unique. Taxonomic studies show that the marine bacteria which have so far been studied fit well into genera which have already been defined. It should be remembered, however, that less than 1% of the bacteria observed in seawater and marine mud by microscopy grow under laboratory conditions. It is therefore quite possible that the organisms so far examined are not representative of the indigenous flora.

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