Numerical Taxonomy of Some Bacteria Isolated from Antarctic and Tropical Seawaters

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Abstract

PFISTER, ROBERT M. (Lamont Geological Observatory, Palisades, N.Y.), AND PAUL R. BURKHOLDER. Numerical taxonomy of some bacteria isolated from Antarctic and tropical seawaters. J. Bacteriol. 90:863–872. 1965.—Microorganisms from Antarctic seas and from tropical waters near Puerto Rico were examined with a series of morphological, physiological, and biochemical tests. The results were plotted on punch cards, and similarity matrices were computed with a program for an IBM 1620 computer. When the matrix was reordered by use of the single-linkage technique, and the results were plotted with four symbols for different per cent similarity ranges, nine groups of microorganisms were revealed. The data suggest that organisms occurring in different areas of the open ocean may be profitably studied with standardized computer techniques.

Increased interest in the use of the Adansonian principle of equal weight for characteristics used to describe organisms (Sokal and Sneath, 1963; Rogers and Fleming, 1964; Quadling and Colwell, 1964; Colwell and Mandel, 1965; Lockhart, 1964) has led us to examine bacteria obtained from the open ocean with similar techniques.

Numerous physiological, morphological, and biochemical characteristics were determined for cultures isolated from Antarctic seas and from tropical waters in the vicinity of Puerto Rico. We have used this information and computer techniques to describe a system which appears to be suitable for the study of small or infinitely large marine populations. There has been much effort devoted recently to the numerical approach in microbial taxonomy, especially among microbiologists who recognize the need for improved techniques. The merits and faults of the numerical approach to problems of bacterial classification have been well covered in recent papers (Sokal and Sneath, 1963; Quadling and Colwell, 1964).

Materials and Methods

Organisms. The 151 microorganisms used in this study were isolated from the Antarctic Sea and from waters in the vicinity of Puerto Rico. Cultures were purified by restreaking, and maintained on a medium containing the following (per liter): Trypticase (BBL) or N-Z-Case, 2.0 g; Soytone (Difco), 2.0 g; yeast extract, 1.0 g; vitamin B12, 1.0 µg; aged seawater; agar (if desired), 16 g.

The pH was adjusted to 7.0 prior to autoclaving. Isolation of Antarctic microorganisms was carried out in two ways. (i) If the desired organisms were psychrophilic and still able to withstand and grow at warmer temperatures (e.g., 20 to 40°C), then a standard pour-plate technique of melted agar at 47°C and incubation at 5°C was used. (ii) When obligate psychrophiles (microorganisms not able to grow above 10 to 15°C) were desired in addition to the other types, their isolation was carried out by a procedure which maintained the seawater sample below 10°C at all times. Obligate psychrophiles were isolated by quantitatively depositing the sample on the surface of a precooled seawater agar plate, spreading with a cold glass rod, and incubating at 5°C for periods up to 2 weeks. Examination of the agar surface for the presence of clones was made in the cold with a stereomicroscope. Bacteria were isolated from tropical waters by the common pour-plate technique.

Media and tests. To determine the carbohydrate fermentation of these marine bacteria, a 1.0% carbohydrate medium (see Fig. 2 for specific carbohydrates) was prepared according to Skerman (1959), with the seawater medium previously described, but with K2HPO4 added in the amount of 0.05 g per liter and use of agar at the rate of 5.0 g per liter. The solutions of carbohydrates were sterilized by Seitz filtration and added aseptically to 1-liter batches of melted base medium. All media were preincubated for a minimum of 72 hr to check for sterility. Each organism was inoculated in each test medium in duplicate with a butt stab. Samples were prepared for anaerobic fermentation by covering with sterile mineral oil. With the tropical bacteria, the test medium was covered

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immediately after inoculation, because a few of the water organisms utilized the carbohydrates aerobically so rapidly that any anaerobic test was spoiled before leaving the inoculation area. All cultures used for inoculum were 48-hr transfers, actively growing on seawater agar. Incubation was at 25°C, except where otherwise specified.

Indole production was determined (Skerman, 1959) by use of 1% Tryptone (Difco) in the seawater medium.

Starch hydrolysis was carried out by preparing a 0.2% solution of soluble starch in the basal agar medium. Cultures were streaked on the surface of the agar and incubated for 1 week, at which time a dilute iodine solution was poured over the surface, and the cleared zones were recorded.

Gelatin hydrolysis was carried out by use of seawater agar plates containing 0.4% gelatin. After incubation, colonies were flooded with a 15% acid HClO4 solution (Skerman, 1959) to make visible the zones of hydrolysis.

Methyl red and Voges-Proskauer tests were tried according to Skerman (1959), with the substitution of seawater for the diluent, but the high percentage of KH2PO4 formed an insoluble precipitate and the phosphate had to be reduced to 0.35 g per liter.

Citrate utilization as a source of carbon for growth was determined with a medium containing synthetic seawater (Lyman and Fleming, 1940) containing the following (per liter): KH2PO4, 50 mg; Fe(NH4)2(SO4)2·6H2O, 7 mg; N-Z-Case, 0.4 g; and sodium citrate, 3.0 g. To ensure that growth in the medium was due to citrate utilization and not the substitution of amino acids as a carbon source, the level of 0.4 g per liter of N-Z-Case was determined experimentally to be suitable for demonstrating increased growth with added citrate.

Amino acids were tested as a carbon source by inoculating a medium of synthetic seawater containing, per liter, KH2PO4, 0.025 g, and N-Z-Case, 4.0 g.

Hydrolysis of triolein and tributyrin was detected by dispersing the fats in a melted 0.5% neutral agar solution (10% fat) and autoclaving. In preparation for use, the fat-agar emulsion was melted and added aseptically to the melted basal seawater agar (1 ml of fat emulsion per 20 ml of basal medium), and then shaken and dispersed to plates. The organisms were streaked on the surface, incubated up to 2 weeks and examined for a clearing zone (Tendler and Burkholder, 1960), and after staining with Sudan B solution to help intensify the clearing zones.

Nitrate reduction was detected by the method outlined in the Manual of Microbiological Methods (Society of American Bacteriologists, 1957), by use of seawater broth with 0.1% KNO3.

The Kovals oxidase test was performed according to the method of Koval (1956). The cytochrome oxidase test of Gaby and Free (1958) was used as described, except for the addition of the reagents to an agar slant in preference to broth culture. Catalase activity was qualitatively detected by adding a drop of H2O2 to an agar slant culture.

Litmus milk was prepared at 0.33% of the recommended concentration in seawater with the addition of 1 μg per liter of vitamin B12. Sterilization of this medium was done by a 3-day successive tyndallization (5 min of free flowing steam each time), with incubation at 25°C between steam treatments. Prepared medium was tested for sterility by preincubation for at least 72 hr prior to use.

Ammonium nitrate was tested as a nitrogen source by use of a medium of seawater with additions of the following (per liter): KH2PO4, 0.05 g; NH4NO3, 5.0 g; glucose, 4.0 g; fructose, 4.0 g; and sodium citrate, 4.0 g.

H2S production was determined by a technique modified from Skerman (1959), with the following medium (per liter): peptone, 10 g; cystine, 0.1 g; Na2S04, 0.5 g; seawater; and vitamin B12, 1 μg. The pH was adjusted to 7.0. Sterile lead acetate paper strips were placed at the top of the tube between the plug and the glass. Darkening of the paper strips at any time during incubation was considered a positive indication for the production of H2S.

Sensitivity of the organisms to six antibiotics—bacitracin, novobiocin, oleandomycin, penicillin, tetracycline, and viomycin—was determined by use of standard Difco sensitivity discs impregnated with "medium" levels of concentration for each antibiotic. Cultures used to form the seed layer for testing the sensitivity discs were grown in seawater broth on a rotary shaker at 25°C overnight, and then layered on the surface of poured agar plates. The sizes of zones of inhibition were not taken into account, but inhibition was recorded as either present or absent. At the same time, a solution of pteridine 0.129 (Shewan, Hodgskiss, and Liston, 1954) was tested in moistened paper discs placed on the seeded plates, to aid in the differentiation of Vibrio and Pseudomonas.

Microscopic and macroscopic observations of morphological and cultural characteristics were made by use of seawater agar slants, isolated clones on streak plates, and, where necessary, in seawater broth. Subjective decision was made where colony color was noted or diffusable pigments detected. Subjective decision was also used in determining the amount of growth in any broth culture, based upon the development of relative turbidity in a given time, which was arbitrarily scaled 0, 3, or 5, with 0 equal to slight growth, 3 equal to fair growth, and 5 good growth.

Gram stains were tried according to several methods, e.g., Hucker modification and Burke, Kolopoff-Beeran modification (Society of American Bacteriologists, 1957), with the result that no preference for a method could be made. Measurements of the cell dimensions were taken from
Gram-stained preparations, whereas configuration of the cells (morphology) was determined from a wet mount by use of phase-contrast optics. Flagellation of bacteria was determined by the method of Leifson (1960).

**Numerical analyses.** To compute a per cent similarity ($%S$; Sokal and Sneath, 1963) for each isolate (OTU) compared with all other isolates, two types of calculations were made. One method was that of Sneath (1957), in which negative matches were excluded, and the other was the technique of Sokal and Michener (1958), in which negative matches were included in the numerator. These methods are computed as follows. According to Sneath (1957),

$$%S = \frac{N_{sp}}{N_{sp} + N_d} \times 100$$

and according to Sokal and Michener (1958),

$$%S = \frac{N_{sp} + N_{sn}}{N_{sp} + N_{sn} + N_d} \times 100$$

where $%S$ = similarity coefficient, $N_{sp} =$ number of similar positive matches, $N_{sn} =$ number of similar negative matches, and $N_d =$ number of dissimilar matches. The raw data were punched onto standard IBM cards in a manner described by Colwell and Liston (1961).

The program was written in Fortran II in conjunction with an IBM 1620 computer equipped with an accessory disc memory storage unit.

Each per cent similarity was computed as three digits, or to a 0.1% accuracy. Grouping of the various OTU values was done with the method of single-linkage clustering (Sokal and Sneath, 1963),

**Fig. 1.** Computed per cent occurrence of morphological characteristics, pigment production, and growth phenomena based upon the combined marine population of 150 microorganisms.
which first clusters strains most closely related with the highest similarity coefficients. Then, by lowering the level of inclusion by predetermined amounts of equal magnitude, it permits the other members to join. In this manner, one can find the exact value at which all members of the matrix become numerically united.

**Results**

Figures 1, 2, 3, and 4 represent a compilation of various taxonomic measures made upon a population of 151 marine microbes taken from either the cold Antarctic sea, or from warm tropical waters in the vicinity of Puerto Rico.

In Fig. 1, a bar graph shows the various categories of morphology, pigment production, and growth observed during this study. Each bar on the graph is the computed percentage of occurrence of the character in question based upon the total number of organisms examined for that character. The data in Fig. 1 were determined for a total of 151 microbial isolates examined for each character; 114 isolates were derived from cold Antarctic waters and 37 from tropical waters near Puerto Rico.

The bar graph in Fig. 2 shows the results of tests employed to help describe this marine population. The total number of organisms studied in this series of tests was 151, except that cellobiose utilization was calculated from a total of 115 organisms, and tributyrin, triolein, and pteridine 0/129 reactions were based upon tests made with a total of 91 cultures.

A partial list of characters for the Antarctic segment of the population is shown in Fig. 3. The ability of the 37 Puerto Rican isolates to use carbohydrate can be seen in Fig. 4.

The ability of the combined population (151 cultures) to use aerobically and anaerobically seven selected carbohydrates in cumulative combination is shown in Table 1.
A diagonal matrix of similarity coefficients (\(\%S\)) for 88 Antarctic bacteria, 6 tropical water isolates, and 2 yeast cultures was computed according to the formula of Sokal and Sneath (1958), which includes negative matches and is based upon the tests made of each isolate. The matrix (M) generated consisted of 4,560 \(\%S\) values comparing each member OTU (N) with every other \([M = N(N - 1)/2]\). This matrix is random, since no attempt was made to organize or position any of the member OTU values. The matrix means little, and an attempt to rearrange the order of the member isolates into meaningful groups was made by use of the single-linkage cluster technique (Sokal and Sneath, 1963).

The rearranged matrix was partially plotted, with four symbols to represent four numerical ranges (Fig. 5). The total range of plotting, 80 to 100\(\%S\), was decided upon after sorting the random matrix, because it was found that between the 80 and 84.9\%S level all the members of the test became numerically united. The four symbols divide the range into 5\% groups. The selection of 5\% groups was arbitrary and might be adjusted to smaller values to increase the resolution of the technique.

The single-linkage sorting procedure revealed nine major groups (clusters) which have been numbered from the smallest and farthest removed groups (lower right hand corner of the diagonal in Fig. 5) to the largest single cluster (upper left of Fig. 5), which represents the majority of microorganisms included in the analysis.

When the various characters used to calculate the \(\%S\) matrix are plotted according to the groups shown in Fig. 5, it becomes obvious that each cluster has a distinct pattern of characters.

Table 2 indicates the geographic areas of the three U.S. Navy Eltanin (National Science Foundation research vessel) Antarctic cruises from which the cold-water isolates were taken. The table shows the groups which were determined by inspection of the sorted matrix (Fig. 5).
and the number of purified isolates from each cruise in each group.

The bacteria in each group were partially identified in the classical manner (Bergey's Manual), and were found to be arranged in the following way: Group I, a small cluster of bacteria belonging to the family Micrococcaceae; Group II, mainly gram-positive cocci or coccobacillary forms which are nonmotile, nonsporeforming, and nonearbohydrate-using organisms; Group III, members of the family Pseudomonadaceae which do not attack carbohydrates and may be members of the Proteobacter, Alginomomas, or Mycoplana genera; Group IV, bacteria from the tropical waters in the vicinity of Puerto Rico, which are all gelatin liquifiers and methyl red-positive marine forms of the Pseudomonadaceae (appear to be similar to P. ichthyothodes as described in Bergey's Manual); Group V, members of the family Achromobacteraceae and probably members of the genus Achromobacter; Group VI, members of the genus Flavobacterium; Group VII, Pseudomonas types which liquify gelatin, attack carbohydrates aerobically and anaerobically, and hydrolyze starch; Group VIII, representatives of Pseudomonas species which liquify gelatin but, except for one, do not hydrolyze starch and do not use mannitol, lactose, and mannose; Group IX, also Pseudomonas species, but many of these digest starch and all show good carbohydrate utilization, except for the aerobic use of mannose.

The results of values computed according to Sneath's (1957) method, in which negative matches were excluded, gave a matrix identical to that plotted by including negative matches. The only difference noted was in the value of the %S computed.

**DISCUSSION**

The purpose of this study was to establish a foundation for studying a marine microbial population on a large scale, with the eventual application of numerical taxonomy to act as a method of organizing or separating members of a vast unknown group. No attempt was made at this time.
to identify explicitly any members of this group of marine microbes, but only to gather as much useful information as possible for taxonomic purposes. From this kind of population survey, we may learn what can be of taxonomic importance within any given group, with the result that it may be possible to weight accurately and legitimately the occurrence of characteristics used in a numerical approach to classification. There has been some discussion (Sokal and Sneath, 1963)
concerning the merits of this procedure, and since little is known about the diagnostic properties of large groups of marine microbes, it has seemed worthwhile to make this study.

The microbial population examined thus far consisted of 114 isolates from Antarctic regions and 37 isolates from tropical waters near Puerto Rico. The methods of isolation varied, resulting in a collection of both obligate psychrophilic organisms (not growing above 10 to 15°C) and organisms capable of growth at higher temperatures. The majority of cultures tested from the Antarctic were of the latter type, and were capable of growth in the temperature range from 5 to 35°C. The obligate psychrophiles were handled carefully with respect to their temperature requirements and environment, since short exposure to even 20°C caused death. The initial isolation of some of these psychrophiles was slow, and plates were examined in the cold up to a 2-week incubation period. When pure cultures were isolated, it was found that luxuriant growth occurred in 48 to 72 hr at 5°C. Careful selection of clones was imperative because many of the small colonies are translucent and can only be seen when using a stereomicroscope to observe colonies on the agar surface.

The majority (96.2%) of the microbes examined were found to be gram-negative single rods with one polar flagellum. This assemblage probably belongs in the group of pseudomonads. Figure 1 shows the disposition of the various forms found in the population, demonstrating a preponderance of cultures containing single cells and diploforms rather than chains. Some of the cultures (8%) were pleomorphic during active growth when examinations were made, whereas many more became pleomorphic late in their growth cycle.

There was some incidence of curved rods among the bacteria tested, and, since some of them were gram-negative with a single polar flagellum and sensitive to the pteridine (0/129) compound of Shewan et al. (1964), they are presumed to be Vibrio types. Two of the Antarctic isolates were yeast cultures, which were included in the testing procedure with no further distinction or attempt at identification.

Figure 2 shows the various biochemical tests that were made, and the resulting per cent of reactions. The six antibiotic-sensitivity tests showed a wide variance of these marine organisms in their susceptibility, with only one Antarctic and one tropical culture being susceptible to all six compounds.

Antibiotics have not generally been considered as a tool for taxonomic work because of microbial adaptability and for various other reasons. It is doubtful, however, that the population of marine microbes had experienced prior contact with the antibiotic compounds, and perhaps the determination of antibiotic sensitivity may be of some value in taxonomic studies. This is especially true when these tests are considered principally as characters to be used in numerical taxonomic separations. The inclusion of such data in a numerical scheme for taxonomy may be of significance in the ultimate assignment of any members of the population into appropriate phenons.

Examination of the results (Table 1) showing the percentage of total organisms able to ferment cumulatively the seven carbohydrates in the tests suggests in general that both the Antarctic and tropical water species are able to ferment aerobically or anaerobically a variety of carbohydrates. In addition, it can be noted that once carbohydrate utilization is recorded (e.g., glucose fermented) the successive use of another carbohydrate (e.g., glucose plus fructose) may occur with increasing probability. This is most easily seen in the last column of Table 1 which shows all seven carbohydrates are being fermented by the same organisms, and yet this group includes about 30% of the population tested.

There were some distinct differences between the Antarctic and Puerto Rican collections, other than expected temperature requirements. None of the 114 Antarctic specimens was able to synthesize indole, whereas 46% of the tropical isolates gave a positive test for the compound. In addition, the ability of the bacteria from tropical waters to ferment aerobically or anaerobically the various carbohydrates of the test was slightly greater than that of the Antarctic specimens. There may be other undetected differences between these populations, but it would be desirable to increase the number of specimens, particularly the tropical isolates, before definitive statements can be made. The significance of the difference in indole production is not understood at the present, but it may reflect a biochemical alteration in tryptophan metabolism characteristic of the populations.

That 83% of the organisms should require seawater for growth and 17% show ability to grow also in distilled water media is of interest. The presence of nutritive materials in the shallow waters near Puerto Rico must certainly influence the microbial flora of the littoral and neritic habitats. Throughout the varied marine environments of the world oceans, one expects to find complex ecological relationships exerting profound influences upon the types of microorganisms which comprise the microfloral and faunal communities.

We are endeavoring in this program to develop
a system for storage and recall of quantities of
data concerning large if not infinite populations.
Because of the burdensome nature of this type of
research, modern methods of data storage and
processing are being used. All data records are
kept on IBM punched cards in a manner similar to
that used by Colwell and Liston (1961) and
on "Keysort" needle punch cards prepared for
us by the Royal McBee Co., New York, N.Y.
Using these tools, we can now survey populations
of microorganisms which heretofore could not be
studied.

A computer technique with the principle that
every feature should have equal weight in
determining its taxonomic position was used to
analyze a population of marine microorganisms.
The population of microorganisms was composed of
bacteria, with the exception of cultures 148
and 167 which were yeast; these were included
so we could observe whether they would cluster
with any other known group. Examination of Fig. 5 shows the two yeasts with a fairly high %S
value for each other, but not grouped with any
of the other nine major clusters.

There are a number of conclusions that can be
derived from these studies. Most important is
the fact that this technique of computer analysis
appears to be a practical method for studying
very large collections of unidentified marine bacte-
ria.

With a suitable data-storage program, the
computer technique can be used constantly to
calculate the %S value of organisms which are
added to the collection provided the required
number of definitive tests have been made, and
in this way it becomes possible to place new en-
tries in their appropriate clusters. These tech-
niques carried out with a computer on board an
oceanographic vessel would enable the investi-
gator to remain at sea for long periods and make
a systematic appraisal of marine microbial popu-
lations from one geographic location to another.
In this manner, much information about the
microbiology of the sea could be learned readily.
There are probably many uses for this kind of
information. Certainly it would be significant to
have investigations about the food chain, roles of
microorganisms in the productivity, and the
transformation of organic and mineral matter in
the world oceans. It is possible that certain kinds of
bacteria are indicators of temperature, pH, or
salinity values, but without some kind of sys-
tematic study, their presence and usefulness in
studies of the marine environment will not be
appreciated.

Examination of Table 2 shows clustering of
the bacterial isolates with the cruise from which
they were taken, Eltanin 9, 11, or 12. This
clustering may be the result of either the geo-
graphic origin of the isolates, since each cruise
was made in a different area, implying that there
may be different populations in different areas,
or it may be related to the seasons, because each
cruise was made at a different time, implying some
temperature relationships responsible for the
Grouping. There are other factors which may
influence this kind of study, such as local weather
conditions, proximity to land, pH, salinity,
available substrates, and depth from which the
cultures were obtained. Sampling procedures
must be carefully regulated and many samples
need be collected to obtain a representative popu-
lation in any given area.

A large-scale, well-planned, and coordinated
program is required to learn valuable information
about the microbiology of the sea. It is believed
that the numerical approach to taxonomy (identi-
fication) with the capability of massive data
storage will be of practical value for investigations
of large populations in the world oceans.

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