Properties of Bacteria Isolated from Deep-Sea Sediments

M. M. QUIGLEY AND R. R. COLWELL

Department of Biology, Georgetown University, Washington, D.C. 20007

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Thirty-eight isolates were subjected to taxonomic analysis by computer. Of the 38 isolates, 31 were from sediment samples collected at depths from 9,400 to 10,400 meters in the Philippine and Marianas Trenches of the Pacific Ocean, and 7 cultures were from seawater samples collected at various depths from surface to 4,000 meters and from several locations in the Pacific Ocean. A total of 116 characteristics were determined for each isolate, coded, and transferred to punch cards. Similarity values were obtained by computer analysis, with the use of two recently developed computer programs. Five distinct phenetic clusters were observed from the numerical analyses. Four of the clusters were identified as species of the genus *Pseudomonas*, and one, as an aerogenic species of *Aeromonas*. Group IV was identified as pigmented *Pseudomonas fluorescens*, and the major cluster, consisting of groups I and II, which merged at a species level of similarity, was treated as a new species of *Pseudomonas*. The 38 strain data were compared with data for 132 marine and non-marine strains previously subjected to computer taxonomic analysis. The baro-tolerant deep-sea strains, with the exception of the deep-sea *P. fluorescens* isolates, clustered separately from all other marine strains.

Since the application of computer analysis of taxonomic data for the bacteria was first proposed by Sneath (28), many investigators have made use of Adansonian principles to describe and classify bacteria. Various authors have examined the techniques and have proposed modifications of the methods for Adansonian classification (1, 2, 27, 30). Adansonian principles were first applied to marine microorganisms by Colwell and Liston (5), and other investigators have since similarly studied the marine bacteria (9, 11, 21). A clarification of the relationships amongst the marine bacteria has resulted from these and subsequent studies. Furthermore, the correlation of deoxyribonucleic acid base ratios with Adansonian analysis (6, 7, 26) has provided confirmation of many of the numerical taxonomy results. The relationships among strains of the phenons obtained in computer analysis and of the various bacterial genera and species examined (18) provide a basis for application of these principles in studies of the classification and identification of unknown isolates (21, 22).

Bacteria isolated from deep-sea sediment samples, obtained at depths greater than 6,000 meters, have not been extensively studied for several reasons, the most obvious being because of sampling difficulties. Therefore, few data on

the physiological, morphological, and biochemical characteristics of the deep-sea bacteria are available. Observations made during the *Galathea Expedition* of 1950-1952 demonstrated for the first time the presence of living bacteria in sediment samples obtained at depths greater than 6,000 meters (33-35, 37). Prior to this time, most microbiologists questioned whether conditions of temperature, pressure, oxygen, and nutrient levels would permit the existence of bacteria at depths greater than 6,000 meters (32). Initially, investigators were concerned primarily with establishing the existence and the numbers of bacteria extant at great depths (14, 37). Some attention was directed toward the biochemical and physiological capabilities, such as starch hydrolysis, nitrate reduction, heat and pressure sensitivity, sulfate reduction, ammonification, and nitrification of the bacterial populations in deep-sea sediments (37), and toward the effects of in situ pressures on deep-sea bacteria and bacterial systems (36). There have been, however, no concomitant studies on the taxonomy of pure cultures isolated from sediment or seawater samples of the deep sea.

The present study concerns the characterization, identification, and classification of bacterial cultures isolated from sediment samples obtained

from the Philippine Trench (9,854 and 9,443 meters) and the Challenger Deep (10,373 meters) in the Marianas Trench of the Pacific Ocean. This study of the morphology, physiology, and biochemistry of the pure cultures represents the first such extensive compilation of information for deep-sea bacteria.

MATERIALS AND METHODS

The cultures included in this study are listed in Table 1. Thirty-one cultures (prefix A, B, and C) were isolated in November 1964, during the *Dodo Expedition* (isolated by R. Y. Morita, Oregon State University, Corvallis, Ore., and C. E. ZoBell, Scripps Institution of Oceanography, La Jolla, Calif.).

All culture with the prefix A or B were isolated from samples of Philippine Trench sediment (*Expedition Dodo* Stations no. 280 and 281, respectively), and cultures with the prefix C were from the Challenger Deep (Marianas Trench) sediment samples (Station no. 282). The sampling information for the *Dodo* cultures is summarized in Table 1.

The *Dodo* cultures were isolated from the top of the sediment cores taken at the given depths. The letter signifies the core identification, the number, and the sequence of isolation. The M (see Table 1) signifies the M medium or Marine Agar (Difco) employed for the isolation, which had the following composition: peptone, 5.0 g; yeast extract, 1.0 g; ferric citrate, 0.1 g; sodium chloride, 19.4 g; magnesium chloride, 8.8 g; sodium sulfate, 3.24 g; calcium chloride, 1.8 g; potassium chloride, 0.55 g; sodium bicarbonate, 0.16 g; potassium bromide, 0.08 g; strontium chloride, 0.034 g; sodium fluoride, 0.0024 g; ammonium nitrate, 0.0016 g; disodium phosphate, 0.008 g; agar, 15.0 g; and distilled water, 1 liter. The P signifies P medium, the SDB psychrophilic medium (10) containing: Polypeptone (Difco), 5.0 g; yeast extract (Difco), 3.0 g; Rila Marine Mix, a synthetic seawater salt mixture (Rila Products Co., Teaneck, N.J.), 6.0 g; sodium chloride, 21.0 g; glucose, 1.0 g; succinic acid, 0.3 g; Tween-80, 0.5 ml; agar, 15.0 g; and distilled water, 1 liter. The pH of the media was adjusted to 7.3 with sodium hydroxide. Isolation procedures were carried out at ship ambient temperature and 1 atm of pressure.

The seven cultures isolated from seawater samples taken at various depths and locations in the Pacific are listed with location of isolation (Table 1). The sampling procedure for the R/V *Acona* Expedition seawater isolates (see Table 1) was as follows. All isolates obtained above 200 meters were taken with the J-Z water sampler (32), and below 200 meters, with Teflon-coated Nansen bottles sterilized with 95% ethyl alcohol. Samples were removed from the J-Z water sampler or the Nansen bottle as soon as it was taken aboard ship. Immediately after the water samplers were hauled up, the seawater (8 ml) was mixed with 2.0 ml of sterile medium (*vide infra*), and incubated at 5 C until turbid, after which 0.2 ml was added to 75 ml of fresh medium and placed on a shaker until slightly turbid. A loopful was transferred, and the transfer was repeated for isolation

after growth was observed. Colonies were picked and restreaked. All isolation procedures were performed at 5 C and 1 atm of pressure.

The medium used for isolation of the R/V *Acona* cultures listed in Table 1, except 91-1000L and 66-0, contained: yeast extract, 0.5 g; K_2HPO_4 , 0.1 g; succinic acid, 1.0 g; NaCl, 20.0 g; $MgSO_4$, 0.8 g; $CaCl_2$, 0.1 g; $(NH_4)_2SO_4$, 0.1 g; $FeSO_4$ solution (1 mg/ml), 1 ml; trace elements solution ($ZnSO_4$, 0.7 mg/ml; $CuSO_4$, 2.5 μ g/ml; $MnSO_4$, 1.5 μ g/ml; $CoSO_4$, 1.5 μ g/liter; and MoO_3 , 0.5 μ g/liter), 1.0 ml; and distilled water, 1 liter; the pH was 7.2. The medium of isolation for culture 91-1000L was as listed except that 0.5 g of lactose replaced the yeast extract. The medium of isolation for culture 66-0 contained 0.5 g of glycerol instead of yeast extract.

The *Dodo* and R/V *Acona* cultures, after being packed in ice, were delivered to the Georgetown University laboratories by R. Y. Morita in April 1965. Stocks were prepared from the pure cultures by streaking for single colony isolation, serially, three times. The stock cultures were stored at 5 C. Before each testing procedure was carried out, the cultures were examined for viability and purity.

The maintenance medium employed throughout the studies reported here contained: yeast extract (Difco), 0.3%; Proteose Peptone (Difco), 1.0%; and a salt solution consisting of (per liter of distilled water) sodium chloride, 2.4%; potassium chloride, 0.07%; magnesium chloride, 0.53%; and magnesium sulfate, 0.7%; adjusted to pH 7.2 to 7.4 with sodium hydroxide.

The tests carried out on each culture included: morphology under phase contrast, determined on wet-mount preparations of 48-hr broth cultures; flagella staining according to the method of Leifson (15); Gram staining according to the Kopeloff-Beerman modification (29); colony characteristics on agar plates (2% agar added to the maintenance medium) after incubation for 4 to 6 days at 25 C; degree of turbidity (5) produced in a broth culture after 48 hr of incubation at 25 C; growth at temperatures of 0, 5, 7, 8, 10, 15, 25, 30, 35, and 37 C; growth at added NaCl concentrations of 0.0, 0.5, 3.0, 5.0, 7.0, and 10.0%; fluorescence (20); and seawater requirement for growth [three serial platings onto agar plates consisting of: yeast extract (Difco), 0.3%; Proteose Peptone (Difco), 1.0%; and Ionagar (Oxoid), 2%; the pH was adjusted with tris(hydroxymethyl)amino-methane (Tris) buffer.]

Susceptibility of the organisms to the following antibiotics was determined: penicillin, 10 units; dihydrostreptomycin, 10 μ g; chloramphenicol, 30 μ g; erythromycin, 15 μ g; kanamycin, 30 μ g; chlorotetracycline hydrochloride, 30 μ g; novobiocin, 30 μ g; polymyxin B, 30 μ g; oxytetracycline, 30 μ g; and tetracycline, 30 μ g. Susceptibility to the vibriostat 0/129 (24) was also determined. BBL Sensi-Disks were used in all cases except for the 0/129, in which case crystals were sprinkled on the inoculated agar plate. Susceptibility was scored as present or absent; no further quantification according to diameter of the zone of inhibition was made.

Other tests performed included: methyl red; indole; Voges-Proskauer; citrate (Simmons); catalase; starch

TABLE 1. Culture numbers and sampling data for isolates included in this study^a

Culture no.					
A1M	C5M	C2P			
A1M #2	C6M	C3P			
A2M	A1P	C4P			
A4M	A2P	C5P			
A5M	A3P	C6P			
A6M	A5P	C7P			
A7M	A6P	66-0 ^b			
B3M	B1P	81-4000			
C1M	B2P	83-4000			
C2M	B3P	91-1000L			
C2M #2	B5P	91-2000			
C3M	B6P	115-400			
C4M		116-40			

Dodo Isolates					
Date	Station no.	Core no.	Latitude	Longitude	Corrected depth (meters)
25 November 1964	280	A	10° 10'N	126° 40'E	9,854
25 November 1964	281	B	10° 3.8'N	126° 40'E	9,443
28 November 1964	282	C	11° 20'N	142° 19'E	10,373

R/V Acona Cruise Isolates				
Isolate no.	Date	Latitude	Longitude	Depth (meters)
66-0	February 1965	51° 40.1'N	127° 55.6'W	0 ^c
81-4000	10 February 1965	16° 0'N	156° 27'W	3,946
83-4000	12 February 1965	14° 0'N	155° 57'W	3,938
91-1000L	28 February 1965	33° 6'N	144° 12'W	985
91-2000	28 February 1965	33° 6'N	144° 12'W	1,970
115-400	22 March 1965	58° 2.7'N	134° 52'W	400
116-50	23 March 1965	58° 21.3'N	134° 47'W	50

^a Dodo expedition stock culture numbers assigned by C. E. ZoBell, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, Calif., and R/V Acona stock culture numbers, by S. D. Burton, Institute of Marine Science, University of Alaska, College, Alaska.

^b Isolated by D. K. Button, Institute of Marine Science, University of Alaska, College, Alaska.

^c Surface isolate.

hydrolysis; nitrate and nitrite reduction; gelatin liquefaction; peptonization of litmus milk; growth in Vitamin Free Casamino Acids (29); lecithinase on egg yolk-agar (31); lipase activity in Tweens 20, 40, 60, and 80 (25); oxidase (13); arginine, lysine, and ornithine decarboxylase (19); glucose, galactose, lactose, maltose, mannitol, and sucrose utilization (aerobic and anaerobic; 12); and iodoacetate inhibition of acid production in a glucose medium (8).

All media, with the exception of Paton's medium (20), were made up with salts solution (maintenance medium, *vide supra*). In most cases, dehydrated media were prepared directly with the salts solution as diluent; however, litmus milk was prepared in distilled water, and a sterile, double-strength salts solution was added (50%, v/v) after sterilization of the litmus milk. The medium for the methyl red and Voges-Proskauer tests was prepared double strength in distilled water and sterilized; a sterile, double-strength salts solution

was then added (50% v/v). Paton's medium was prepared with distilled water as diluent and 0.5% NaCl was added. A total of 116 features were scored and coded for computer analysis.

All data were coded and transferred to IBM punch cards. Data from previous analyses (R. R. Colwell and M. Gochner, *Bacteriol. Proc.*, p. 40, 1963; Colwell, Gochner, and Quigley, *Soc. Ind. Microbiol. Newsletter* 17:20, 1967) were retrieved for comparisons. The computer used for the analysis was the IBM 1620 Model II computer with 1311 Disk Pack System. Similarity values (5) and phenetic clusters (30) were obtained. The feature frequencies (4) and median organism calculations (18) were determined for the cluster output in the course of the computer analysis. The programs written for taxonomic analysis and used in this study were GTP-2 (Georgetown Taxonomy Program 2), GTP-3, GTP-4, and GTP-5. GTP-2 is a program employing a modification of the

highest-link sorting method of Sneath (28), whereby the affinities among strains of an analysis set are employed both to determine groups and to position strains within a group. GTP-3 provides feature frequency of occurrence for clusters, and GTP-4 computes for each cluster the hypothetical median organism. The comparison of each strain of the cluster with the hypothetical median permits selection of a strain with the highest similarity to the computed median as the "typical" strain. GTP-5 output is the full *S*-value matrix ordered by GTP-2. The programs have been documented for the IBM 1620 Computer Users' Group and are available from the author (R.R.C.) or directly from the Georgetown University Computation Center, Washington, D.C.

RESULTS AND DISCUSSION

The phenetic clusters formed at the various similarity levels (*S*-values) are shown in Fig. 1. The groupings obtained at $S \geq 75$ were selected for more detailed study of the organisms and characteristics in each of the phenetic clusters. Five distinct and homogeneous clusters were distinguishable at this level of similarity. A level of $S \geq 75\%$ has been suggested as a possible species level of similarity (5, 18). All of the strains were gram-negative, straight or slightly curved

rods, occurring mainly as single or paired cells with tapered ends, 0.2 to 1.2 μ long and 0.2 to 0.6 μ wide. Colonies on agar were 2 to 5 mm in diameter, were predominantly convex, were opaque, had an entire edge, and were off-white in color. All strains were oxidase-positive, lipolytic (Tween 20 and 60), and sensitive to kanamycin (30 μ g). The strains grew at temperatures ranging from 0 to 30 C, in NaCl concentrations of 0 to 5%, and in Vitamin Free Casamino Acids-salts medium. Four of the strains required seawater, either artificial or natural, added to the basal medium. However, all strains grew well in seawater-based media. The starch hydrolysis lysine decarboxylase, methyl red, and indole, tests were uniformly negative. Table 2 lists other features which were variable among the strains, and shows the frequency of occurrence for all the groups indicated in Fig. 1.

Group I at $S \geq 79\%$ is composed of eight members (AIP, A3P, A5M, C6M, B3M, A6M, B3P, and A5P), all of which were isolated from the deep-sea sediment samples. With the exception of B3P, the strains did not require the addition of natural or artificial seawater for growth, did not produce acid either aerobically or

FIG. 1. Sorted output, indicating groups formed by the GTP-2 highest-linkage sorting analysis.

TABLE 2. Selected features describing the principal clusters

Feature	Group I	Group II	Group III	Group IV	Group V	Group VI
Motile.....	±(0.4) ^a	+(0.8)	+(1.0)	+(1.0)	+(1.0)	±(0.5)
Polar flagella.....	±(0.6)	+(0.8)	+(1.0)	+(1.0)	±(0.5)	±(0.5)
Peritrichous flagella.....	-(0.0)	-(0.1)	-(0.0)	-(0.0)	+(1.0)	-(0.0)
Pteridine (0/129)-sensitive.....	±(0.3)	-(0.0)	-(0.0)	-(0.0)	+(1.0)	-(0.0)
Penicillin (10 units)-sensitive.....	+(1.0)	±(0.4)	-(0.0)	-(0.0)	+(1.0)	-(0.0)
Dihydrostreptomycin (10 µg)-sensitive.....	+(1.0)	+(1.0)	+(0.8)	-(0.0)	+(1.0)	+(1.0)
Polymyxin B (30 µg)-sensitive.....	+(1.0)	+(1.0)	+(1.0)	+(1.0)	-(0.0)	+(1.0)
Glucose oxidative.....	-(0.0)	+(0.9)	+(1.0)	+(1.0)	-(0.0)	+(1.0)
Glucose fermentative.....	-(0.0)	-(0.1)	-(0.0)	-(0.0)	-(0.0)	+(1.0)
Glucose gas.....	-(0.0)	-(0.0)	-(0.0)	-(0.0)	-(0.0)	+(1.0)
Lactose oxidized and fermented.....	-(0.0)	-(0.0)	-(0.0)	-(0.0)	-(0.0)	+(1.0)
Maltose oxidative.....	-(0.0)	-(0.2)	±(0.5)	-(0.0)	-(0.0)	+(1.0)
Maltose fermentative.....	-(0.0)	-(0.0)	-(0.0)	-(0.0)	-(0.0)	+(1.0)
Mannitol oxidized and fermented.....	-(0.0)	-(0.0)	-(0.0)	-(0.0)	-(0.0)	+(1.0)
Sucrose oxidative.....	-(0.0)	+(0.9)	-(0.0)	+(1.0)	-(0.0)	+(1.0)
Sucrose fermentative.....	-(0.0)	-(0.0)	-(0.0)	-(0.0)	-(0.0)	+(1.0)
Glucose and iodoacetate → acid.....	-(0.0)	+(0.9)	+(1.0)	+(1.0)	-(0.0)	+(1.0)
Galactose oxidized and fermented.....	-(0.0)	-(0.0)	+(0.8)	-(0.0)	±(0.5)	+(1.0)
Arginine decarboxylase.....	-(0.0)	-(0.0)	+(1.0)	+(1.0)	-(0.0)	±(0.5)
Voges-Proskauer.....	-(0.0)	-(0.0)	-(0.0)	-(0.0)	+(1.0)	+(1.0)
Citrate utilized.....	-(0.0)	-(0.2)	+(0.8)	+(1.0)	-(0.0)	-(0.0)
Gelatin liquefied.....	±(0.3)	-(0.1)	±(0.3)	±(0.3)	+(1.0)	±(0.5)
Litmus milk peptonized.....	-(0.0)	-(0.0)	±(0.3)	±(0.7)	+(1.0)	±(0.5)
Catalase.....	±(0.4)	+(1.0)	+(1.0)	+(1.0)	+(1.0)	+(1.0)
Lipase (Tween 80).....	+(1.0)	-(0.1)	±(0.5)	+(1.0)	+(1.0)	±(0.5)
Nitrate reduced.....	-(0.0)	±(0.4)	+(1.0)	+(1.0)	+(1.0)	-(0.0)
Nitrite reduced.....	-(0.0)	-(0.0)	±(0.3)	-(0.0)	-(0.0)	-(0.0)
Lecithinase present.....	+(1.0)	-(0.0)	-(0.0)	+(1.0)	-(0.0)	-(0.0)
Fluorescence in Paton's medium.....	-(0.1)	-(0.0)	±(0.5)	±(0.7)	-(0.0)	-(0.0)
7% added NaCl growth.....	+(1.0)	+(1.0)	+(1.0)	±(0.3)	+(1.0)	±(0.5)
10% added NaCl growth.....	+(1.0)	+(1.0)	-(0.0)	±(0.3)	+(1.0)	-(0.0)
Temperature range ^b	0-37	0-37	0-30	0-37	0-37	0-37
No. of strains in group.....	8	13	4	3	2	2

^a Frequency of occurrence.^b Temperatures tested were 0, 5, 7, 8, 10, 15, 25, 30, 35, and 37 C.

anaerobically in carbohydrate media, and exhibited polar flagella. (Only 40% of the strains were actively motile under phase-contrast, but in 63% of the group I cultures polar flagella could be observed electron microscopically by use of staining or by the shadowing-casting technique; see Table 2.) From a consideration of the characteristics, this group was identified as a member of the genus *Pseudomonas*. An hypothetical strain was calculated, and strain B3M was found to be 98% similar to the hypothetical strain (see Fig. 2).

Group II, at $S \geq 79\%$, consisted of 13 strains (A4M, A2P, C3M, C4P, C1M, C6P, C2M #2, C2M, A7M, C5P, A1M #2, C2P, and C3P), all of which were isolated from deep-sea sediment but included representatives of both the Philippine Trench and the Challenger Deep. The organisms in this group were polarly flagellated, oxidative in glucose, and catalase-positive; they

produced acid in glucose in the presence of iodoacetate, and only one strain, C3P, required the addition of natural or artificial seawater to media for growth. Other features shared by members of this group are cited in Table 2. It should be noted that AIM exhibited peritrichously flagellated cells when observed by flagella staining or by electron-microscopy shadowing techniques. The rest of the strains of groups I and II were lophotrichously flagellated (one to three flagella). This cluster was identified as belonging to the genus *Pseudomonas*, and further consideration of the data led us to combine groups I and II into a single species (see Table 2) since the phenons merged at $S \geq 75\%$. The combined groups I and II at 75% S thus encompass 23 strains, BIP through C3P (see Fig. 1).

Group III consisted of four strains (115-400, 81-4000, 83-4000, and 66-0) which were isolated

FIG. 2. Electron micrograph of a chromium-shadowed preparation of isolate number B3M, the median organism of group I. $\times 37,800$.

from seawater samples at depths from 0 to 4,000 meters (R/V *Acona* samples). These strains were polarly flagellated, glucose-oxidative, galactose-utilizing, arginine decarboxylase-positive, and catalase-positive, they liquefied gelatin, and they utilized citrate as sole carbon source. The group also produced acid in the presence of iodoacetate, reduced nitrate, and did not require natural or artificial seawater for growth (see Table 2). The cluster was considered to be a species of the genus *Pseudomonas* and is represented by the organism, 81-4000; $S = 95\%$ to the computed hypothetical median strain. An electronmicrograph of 81-4000, showing morphology and type of flagellum insertion, is shown in Fig. 3.

Group IV, a *Pseudomonas* sp., consisted of the three strains C7P, B5P, and A3P, all of which were deep-sea sediment isolates, polarly flagellated, oxidative in glucose and galactose, arginine decarboxylase-positive, and capable of utilizing citrate as a sole carbon source. Also, the strains in this group produced a green diffusible pigment and acid in glucose with 10^{-3} M iodoacetate added. The strains reduced nitrate, and did not require seawater, artificial or natural, for growth. Strain C7P ($S = 95\%$), when compared with the computed hypothetical median strain, was selected as the most typical of the group. From a comparison of features of this cluster with those of *P. fluorescens*, it is concluded that group IV belongs within that species.

Group V consisted of the two deep-sea sediment isolates (B2P and C4M), $S = 79\%$. These strains were motile, with mixed flagellation; that is, peritrichously and polarly flagellated cells could be discerned both from flagellar stains observed under a light microscope and from chromium-shadowed preparations under an electron microscope. Other features were the following: sensitive to pteridine 0/129, fermentative in galactose, Voges-Proskauer positive, gelatin-liquefying, catalase-positive, and nitrate-reducing. Isolate B2P was selected as the representative strain. The mixed flagellation, sensitivity to 0/129, acid production in galactose under oil (12), and positive Voges-Proskauer reaction might lead one to conclude that these are members of the genus *Vibrio* or anaerogenic *Aeromonas*. However, no other carbohydrates were utilized and the utilization of galactose was slow. From our experience with the Hugh and Leifson technique (12), delayed carbohydrate utilization often leads to a false-positive reading for the carbohydrate-utilization test under oil. Furthermore, Leifson and co-workers, in studies of marine bacteria (16, 17), noted the occurrence of mixed flagellation in both fermentative and nonfermentative straight rods and concluded that diagnosis on the basis of flagellation was extremely difficult.

Buttiaux and Voisin (3) isolated a gram-negative bacterium from mussels and Mediterranean seawater. The features cited for this microorganism correspond almost precisely with the description of strains B2P and C4M. These authors observed both monotrichons and lateral flagella to be present in cultures of their isolate, with the flagellation type seemingly depending upon agar concentration (1 and 2%), sodium chloride concentration (0.5 and 5%), and growth temperature (20 to 22 C and 37 C). They concluded that the bacterium should be placed in the genus *Beneckeia* because of its chitinase activity.

FIG. 3. Electron micrograph of a chromium-shadowed preparation of isolate number 81-4000, the median organism of group III. $\times 46,000$.

The features demonstrated by the group V also appear to resemble closely the strains of the first major group listed in Table 2 of Leifson et al. (17) but not identified by them as to genus and species. The "Gram negative, peritrichous and not obviously Enterobacteriaceae" mentioned by Shewan (23), and cited by him as being of uncertain taxonomic position, may apply to the strains B2P and C4M of this analysis.

In summary, group V joined with groups I and II at $S = 72\%$ in the highest link sort analysis (Fig. 1). Groups III and IV clustered at a similarity value of 70%. Group III-IV and group I-II-V clustered at $S = 69\%$; group VI was identified as an aerogenic *Aeromonas* sp. (Table 2), following the description of Shewan (23). The principal characteristics of the isolates in each of the six phenons are summarized in Table 2.

Deoxyribonucleic acid (DNA) base composition and DNA/ribonucleic acid (RNA) homology studies with the *Dodo* cultures are presently underway.

The coded data from the 38 strains of this analysis were combined with the coded characteristics from 132 previously studied marine strains (R. R. Colwell and M. Gochner, *Bacteriol. Proc.*, p. 40, 1963; Colwell, Gochner, and Quigley, *Soc. Ind. Microbiol. Newsletter* 17:20, 1967). Similarity values and phenetic clusters were calculated for the entire set of 170 isolates.

Of the 132 additional strains, 48 were isolated from water samples collected from the Sargasso Sea, 10 were isolated by R. Hamilton from water samples collected off the pier at the Scripps Institute of Oceanography, La Jolla, Calif., 2 were from Chesapeake Bay water, at Oxford, Md., 3 were isolated by H. Jannasch, Woods Hole, Mass., and 69 were marine strains and type strains from culture collections; the last 69 had been previously studied by computer analysis during an earlier survey (R. R. Colwell and M. Gochner, *Bacteriol. Proc.*, p. 40, 1963).

At $S \geq 76\%$, the deep-sea sediment isolates clustered as groups distinctly separate from the set of 132 additional strains. At $S = 75\%$, group IV, previously identified as *P. fluorescens*, clustered with three *P. fluorescens* strains obtained from other culture collections. At $S = 74$, the combined group I-II, also identified as *Pseudomonas* species, clustered with several known *Pseudomonas* strains, including named, culture collection strains. At $S = 70\%$, the deep-sea strains and the seawater isolates clustered into a single generic group, with the exception, of course, of the 91-2000 and B6P strains, assigned to the genus *Aeromonas*.

The only published work on bacteria isolated from depths greater than 6,000 meters were the

studies of ZoBell (33-35), ZoBell and Morita (37), and Kriss (14). In the report on the *Gala-thea Expedition*, ZoBell and Morita presented data demonstrating the presence of up to 10^6 viable bacteria per gram of wet sediment in sediment samples collected at depths up to 10,200 meters. Investigations of the properties of these bacteria were carried out at in situ temperatures and pressures. However, dilution extinction procedures were followed in examining the deep-sea sediments. The presence of starch hydrolyzers, nitrate reducers, sulfate reducers, ammonifiers, and nitrifiers was demonstrated. Clonal isolates were not studied. Kriss, a Russian scientist, observed 10^2 bacteria per g of wet mud sample in sediment samples collected at depths up to 10,000 meters in the Central Pacific (14), but no definitive descriptions of species isolated are available.

Pfister and Burkholder (21) applied computer techniques to the analysis of 151 bacterial strains isolated from the Antarctic Sea and from waters in the vicinity of Puerto Rico. Their results demonstrated that organisms occurring in different areas of the open oceans may be profitably studied by means of computer techniques, and the data so obtained may well provide a basis for characterizing waters of different geographical areas. However, the data of Pfister and Burkholder are not presented in a manner permitting direct comparisons of the taxa described by them with the data of the present study. A few points may be made, however. The 151 marine strains of the analysis carried out by Pfister and Burkholder formed nine distinct phenetic clusters, not identified explicitly but designated as groups. Group I of Pfister and Burkholder was composed of members of the family *Micrococcaceae*; group II, mainly of gram-positive, nonmotile, non-sporeforming, and carbohydrate-nonutilizing organisms which were not identified further; group III, of carbohydrate-nonutilizing members of the family *Pseudomonadaceae*; Group IV, of bacteria from tropical waters, all gelatin liquefiers and methyl red-positive marine forms similar to *P. ichthyoderms* (*Bergey's Manual*); group V, of organisms probably belonging to *Achromobacter* sp.; group VI, of *Flavobacterium*; and groups VII, VIII, and IX, of organisms considered by Burkholder and Pfister to belong to the genus *Pseudomonas*.

None of these groups, which were rather sketchily described by Pfister and Burkholder, seems to apply to isolates of the present study. Group IV (methyl red-positive and gelatin liquifiers), group VII (liquify gelatin, attack carbohydrates aerobically and anaerobically, and hydrolyze starch), and group IX (digest starch and show good carbohydrate utilization,

except for aerobic use of mannose) do not appear to correspond to any of our phenetic clusters. Group V of this study appears to be similar to group VIII of Pfister and Burkholder (liquify gelatin, do not hydrolyze starch, and do not utilize mannitol, lactose, and mannose). However, the two strains of group V of our study demonstrated mixed flagellation, being both polarly flagellated and peritrichously flagellated cells in a pure culture. Further comparison is not possible with the published data available to us.

Incubation of test media for the deep-sea isolates (except for temperature and growth range studies) was at ambient (20 to 25 C) temperature, and the majority of the cultures grew well between 0 and 35 C. Interestingly, the majority of the cultures isolated from Antarctic waters by Pfister and Burkholder were capable of growth in the temperature range from 5 to 35 C. Although ZoBell and Morita (37) found that many deep-sea bacteria were killed after 10 min of exposure to 30 C, these deep-sea cultures, having been isolated at 20 to 25 C, could be considered to have been selected from the total viable population of the sediments in part on their ability to grow in relatively high temperature range (as compared with the in situ temperature of < 10 C) and in part on their ability to grow at the reduced pressure (1 atm) employed in the isolation procedure. Only one of the groups (*Pseudomonas* group III) did not grow at temperatures above 30 C. Conversely, all strains grew at 0 C, the lowest temperature which was tested. Furthermore, all strains were barotolerant, growing at pressures of 11,000 atm. Terrestrial or freshwater strains do not survive pressures of this intensity (R. Y. Morita, *personal communication*). Thus, the deep-sea isolates examined in the present study would be considered as eurythermic and barotolerant or eubaric.

It is interesting to note that the bacteria isolated from sediments and those isolated from seawater (depths ranging from the surface to 4,000 meters) grouped separately. This is substantiated by the fact that none of the sediment isolates was more than 71% similar to any of the seawater isolates. The separation is further accentuated by the fact that four of the five deep-sea phenons remained separate and distinct even when compared with 132 marine bacteria isolated from specimens collected from the inshore marine environments. The distinction between sediment and water-column bacterial populations has been noted by other investigators (J. Liston and W. J. Wiebe, *unpublished data*). Only one of the 75S-phenons (32), group IV, clustered with any of the previously examined marine and nonmarine strains at a species level of similarity.

The ecological role of the deep-sea isolates in the sediments at the depths of the oceans appears confounded with the lack of an absolute requirement for natural or artificial seawater on the one hand and their eurythermic, rather than obligately psychrophilic, nature on the other. At least three possible explanations offer themselves. First, these organisms are indeed valid representatives of the microbial ecosystem. Second, the isolates may be those which survive the long passage from surface waters, either as animal or plant commensals, and sink to the ocean floor after the death of the host, remaining essentially "dormant" in the sense of being viable but metabolically inactive. Finally, the isolates are both eurythermic and eubaric, active under in situ temperature and pressure and able to survive the decompression and heating which occur in passage from the ocean bottom to the surface during the sampling procedure. Their less sturdy brethren expire under the stress. In situ pressure and temperature studies of the metabolism of these bacteria, i.e., their ability to grow at 0 C under 11,000 atm of pressure, support this hypothesis. Whether these strains are dominant in the deep-sea sediment ecosystem, or merely opportunists, is a question which requires further study.

Some indication of the role that the deep-sea bacteria may play in mineralization and in the nutritional cycles of the sea bottom can be deduced from the frequency of occurrence of given physiological and biochemical features among the strains examined. With the exception of group VI (strains 91-2000 and B6P), little or no carbohydrate activity was noted for the groups. Gelatin liquefaction, lipase activity, and lecithinase activity were relatively common. The strains studied were all oxidase-positive and the majority catalase-positive.

It could be argued that the dominant portion of the sediment consists of anaerobic bacterial forms. However, our samples were taken from the top of the sediment cores, the "mud-water slurry." Deeper core samples may well contain the anaerobic forms, but the mud-water interface would hardly be considered an anaerobic environment. It should be mentioned, however, that all of the cultures grew well in a candle jar under CO₂-enriched atmosphere. Growth under strictly anaerobic conditions was not tested. Anaerobic counts (facultative and obligate anaerobes) carried out by other investigators studying deep-sea sediments indicated that the total number of anaerobes was approximately the same as the total viable aerobes, or, in most instances, less than the aerobic count (37).

Within the generic cluster consisting of groups I-V, group IV has been assigned to the species

P. fluorescens, and the combined groups I and II, to a new species, the description of which will be published elsewhere. Groups III and V have also been assigned to the genus *Pseudomonas* and group VI to *Aeromonas*. Further study of these groups with additional deep-sea isolates should permit species assignment of groups III, V, and VI.

Study and characterization of cultures isolated from the depths of the oceans are severely limited by the difficulties in obtaining and maintaining samples. In relation to the number of square miles of ocean floor and the very few samples which have been taken for study, our information on the marine microorganisms of the deep sea is meagre, at best. The collection of more samples from a wider geographical area should yield better knowledge of the types and numbers of bacteria in sediments and the role these bacteria play in the ecology of the sea floor. Other analyses may also further establish that some species of deep-sea bacteria are unique and distinct from those found in sediment samples collected from shallow waters or the freshwater habitat. On the basis of the present study, however, two genera and at least one species of the deep-sea bacteria are similar to their shallow water or terrestrial counterparts.

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