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A NUMERICAL TAXONOMIC STUDY OF GRAM-NEGATIVE BACTERIA ISOLATED FROM THE ANTARCTIC MARINE ENVIRONMENT

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RESUME

ETUDE EN TAXONOMIE NUMERIQUE DE BACTERIES GRAM NEGATIF ISOLEES DU MILIEU MARIN ANTARCTIQUE

Un total de 144 bactéries isolées d'échantillons d'eau et de sédiments de l'antarctique, et six souches de référence ont été décrites selon 94 caractères biochimiques, morphologiques, nutritionnels et physiologiques. Les résultats ont été analysés par ordinateur en utilisant, pour le calcul de la similitude globale, les coefficients de liaison simple (S_{SM}) et les coefficients de Jaccard (S_J). Les regroupements ont été réalisés par la méthode de la liaison moyenne. A partir du dendrogramme on constate que 140 souches, soit 97 % du total, se sont regroupées en dix phénons formés à plus de 70 % de similitude. Seulement 76 % des bactéries ont pu être identifiées. Ils comprennent des représentants des genres, *Vibrio/Aeromonas* 50 % ; *Alcaligenes* 22 % ; *Pseudomonas* 2 % et *Flavobacterium* 2 %. Les organismes restant n'ont pu être identifiés à des bactéries déjà décrites dans la 8è Edition du *Bergey's Manual of Determinative Bacteriology*. Si quelques unes des souches ressemblent à une espèce nouvellement décrite, sous le nom de *Vibrio fluvialis* sp. nov., les autres représentent des espèces non encore décrites.

INTRODUCTION

Whilst data are available on the distribution and taxonomy of heterotrophic bacteria inhabiting the water column of the polar seas (Pfister and Burkholder, 1965; Kaneko *et al*, 1979), fewer studies have been made of those occurring in the benthic sediments of the Southern Oceans (Herbert and Bell, 1974). During an investigation into the role of bacteria indigenous to benthic sediments, in the cycling of nutrients within the Antarctic marine environment (Tanner and Herbert, 1981), it was observed that a high proportion of the isolates were Gram-negative, rod-shaped and motile by means of a polar flagellum. However, the classification of these bacteria, isolated from the Antarctic marine environment, presented major problems due to the lack of information regarding their basic physiology. As a consequence there was a lack of suitable determinative tests and a high proportion of the isolates could not be fully identified when compared against currently available taxonomic keys. Results from previous studies (Pfister and Burkholder, 1968; Kaneko *et al*, 1979) have shown that members of the genera *Pseudomonas*, *Vibrio* and *Flavobacterium* tend to predominate in low temperature environments such as those found in the maritime Antarctic. Many workers have in addition obtained isolates from marine habitats which do not conform to previously described genera (Cook and Goldman, 1976; Mallory *et al*, 1977), and as a consequence have adopted a numerical taxonomic approach in order more precisely to characterise their bacterial isolates. Much has been written on the advantages of this approach to bacterial taxonomy (Sneath, 1957; Hodgkiss and Shewan, 1968; Goodfellow *et al*, 1976), and, since similar difficulties were encountered with bacteria isolated during the course of this study, a numerical approach was adopted in order to classify these bacteria.

MATERIALS AND METHODS

One hundred and forty four Gram-negative bacteria isolated during a routine sampling programme, at Signy Island, South Orkney Islands, Antarctica were included in this study. Samples of benthic sediment or seawater were collected y SCUBA diver, serially diluted in 3% (w/v) saline, then surface plated onto a variety of media including Tryptone Soya Agar, and Nutrient Agar (Oxoid) with an additional 25 g/ℓ NaCl, and Marine Agar 2216 (Difco). Other isolates were obtained by chemostat enrichment of benthic sediments, using a variety of carbon and nitrogen sources at growth limiting concentrations; (in g/ℓ) glucose, 0.5 to 1.0; sodium lactate, 0.01 to 0.1; glycerol, 0.01 to 0.5; KNO_3, 2.0; and NH_4Cl, 0.003 to 1.5. After inoculation, plates were incubated at 4°C for up to 14 days. Representative bacterial colonies were removed from each plate, purified, then maintained on Marine Agar 2216 slopes, at 4°C. Six type strains were included with these unknown isolates, *Pseudomonas aeruginosa* NCIB 8295, *Ps. fluorescens* NCIB 9046, *Ps. putida* NCIB 9494, *Flavobacterium aquatile* NCIB 8694, *Alcaligens faecalis* NCIB 8156, and *Vibrio marinus* MP-1 ATCC 15381.

General physiological and biochemical properties

In general, characterisation tests were incubated at 10°C for 14 days. All media contained an additional 25 g/ℓ sodium chloride. The isolates were maintained on marine agar slopes, and when required, inoculated into Peptone Water (Oxoid) and incubated for seven days. These cultures were then used to inoculate the appropriate test medium. The following tests were performed as detailed in Cowan (1974); oxidase and catalase; production of phosphatase and urease; hydrolysis of agar, cellulose, starch, gelatin, casein and Tween 80; methyl red and Voges Proskauer reactions; malonate utilization and phenylalanine deamination; nitrate respiration; H_2S production; anaerobic growth; pellicle formation; halotolerance and production of indole from tryptophane. Isolates were also tested for their ability to utilize glucose in Hugh and Leifson's medium (Cowan, 1974) and pigment production on Marine Agar 2216, and Kings A and B media (Cowan, 1974).

Morphology

Motility was observed using the hanging drop technique. The position of flagella was determined by electron microscope examination of gold/palladium shadowed preparations of cells grown in peptone water.

Sensitivity to antimicrobial substances

The sensitivity to various antimicrobial substances in concentrations given were determined by a disc diffusion technique (Cowan, 1974) on Marine Agar 2216. The antibiotics used included erythromycin (10 mcg), novobiocin (5 mcg), cloxacillin (5 mcg), penicillin G (1.5 units) ampicillin (2 mcg), streptomycin (10 mcg), tetracycline (10 mcg) and chloramphenicol (10 mcg), together with the vibriostatic agent 0/129 phosphate (0.1% w/v aq. solution).

Nutritional screening

The isolates were tested for their ability to grow on forty-nine different organic compounds as the sole source of carbon. These were added either to peptone water as described by Cowan (1974) or to the minimal salts medium described by Stanier *et al.*, (1966). Incubation of these tests was at 10°C for 14 days.

Temperature studies

Isolates were tested for their ability to grow in peptone water when incubated at various temperatures, between 0°C and 37°C.

Computer methods

Nearly all the characters chosen existed in one of two mutually exclusive states, these being scored as positive or negative. Qualitative multistate characters such as the oxidation/fermentation of glucose were scored as positive for the character state shown (oxidative or fermentative) and negative for the alternative. Similarly, sensitivity to antimicrobial agents was scored as a positive. The resulting data table for one hundred and forty four Antarctic bacteria, six reference strains and ninety four unit characters was analysed using the Clustan 1C. program (Wishart, 1978). Both the simple matching coefficient (Sokal and Michener, 1958) and the Jaccard coefficient (Sneath, 1957) were measured. Clustering of bacteria was achieved using the unweighed-pair-group-average-linkage (UPGMA) algorithm (Sneath and Sokal, 1974).

RESULTS AND DISCUSSION

All the bacteria included in this study were Gram-negative heterotrophs, and the majority, some 96%, were rod-shaped. 50% of the isolates failed to grow at temperature in excess of 20°C, and were therefore defined, according to presently accepted criteria, as psychrophiles (Morita, 1975). A dominant feature of these isolates was their production of pigments (87.5%) when grown on Marine Agar 2216, and that a large number were motile, usually by means of a single polar flagellum. All the isolates grew well aerobically and gave a positive catalase reaction. Many of these bacteria, approximately 79%, were able to grow fermentatively when incubated anaerobically and a high proportion (70%) were able to respire nitrate under anaerobic condition. Cluster composition varied only slightly when the Jaccard (S_J) and simple matching (S_{SM}) coefficients were used with the UPGMA algorithm. Use of the S_{SM} coefficient which produced five major (>6 members) and five minor (2 - 6 members) clusters is described in detail.

A total of 97% of the isolates clustered at a 70% level of similarity. All of the reference strains and four of the isolates failed to cluster at this level. Since none of the reference strains clustered with the other isolates, they have been omitted from the simplified dendrogram (Figure 1). Closer examination of individual cluster characteristics (Figure 1) revealed that approximately 50% of the isolates resembled members of the genera *Vibrio* and *Aeromonas*: 22% *Alcaligenes*: 2% *Pseudomonas* and 2% *Flavobacterium*. Some 24% of the isolates could not however be readily identified with previously described species listed in the Eighth edition of *Bergey's Manual* (Buchanan and Gibbons, 1974).

Cluster 3 comprised pigmented, motile, rod-shaped bacteria capable of growth at 20°C and above. These bacteria were fermentative in their use of glucose and could respire nitrate to nitrogen gas. None demonstrated an obligate requirement for sodium chloride at marine concentrations. It is probable that these mesophilic organisms were not indigenous to the marine environment. Since there was considerable input of freshwater to the nearshore coastal waters of Signy Island, from the freshwater lakes, and as meltwater during the springtime thaw, these bacteria may be of terrestrial or freshwater origin.

Cluster 4 comprised two sub-groups. All these bacteria were rod-shaped, oxidase positive and demonstrated an obligate requirement for sodium chloride. The larger sub-group comprised aerobic bacteria unable to use glucose and resembling the genus *Alcaligenes*. The smaller group was composed of facultatively anaerobic bacteria capable of using glucose fermentatively and resembling the genera *Vibrio/Aeromonas*. The reason for these two sub-groups clustering together was the high percentage of negative matches between isolates, due to their inability to utilize many of the organic compounds tested.

Bacteria included in clusters 5, 8, 9 and 10 were identifiable with previously described species of *Vibrio* and *Aeromonas* but with some differences. Bacteria comprising cluster 5 were psychrotrophic, pigmented, facultatively anaerobic and used glucose fermentatively. They were rod-shaped, oxidase and catalase positive. They resembled *Vibrio costicola* but lacked an obligate sodium chloride requirement. Cluster 8 comprised bacteria sensitive to the vibriostatic compound 0/129, pigmented, fermentative in their use of glucose, oxidase and catalase positive. They resembled *Vibrio parahaemolyticus* but were unable to use citrate or hydrolise casein or starch. However, two biotypes of *V. parahaemolyticus* are at present recognised and these bacteria may represent a further biotype.

Members of cluster 9 were all pigmented, rod-shaped bacteria, facultatively anaerobic, catalase, oxidase and phosphatase positive. All used glucose fermentatively, were motile and showed no sensitivity to 0/129. One member of this cluster produced a brown water-soluble pigment, a characteristic of *Aeromonas salmonicida*. However, these isolates did not produce gas from carbohydrates or hydrolyse casein. Cluster 10 comprised two rod-shaped bacteria, fermentative in their use of glucose, motile, pigmented, but lacking both an obligate salt requirement and 0/129 sensitivity. Both were oxidase, catalase and phosphatase positive and able to respire nitrate to nitrite. They resembled *Aeromonas hydrophila*.

Cluster 7 contained two rod-shaped psychrophilic bacteria. They were motile, oxidase and catalase positive, using glucose oxidatively. Neither would grow anaerobically. Although they resembled the genus *Pseudomonas*, without knowledge of their GC % ratio, it is not possible to assign them positively to this genus.

Whilst most of these bacteria showed slight differences with previously described species, Lee *et al.*, (1978) have reported the existence of many *Vibrio/Aeromonas* strains, isolated from the marine environment which exhibit characteristics intermediate between those of previously described species. In addition, the Antarctic marine environment with its well documented environmental constraints (Tanner and Herbert, 1981) may well have exerted considerable selective pressure leading to the evolution of bacteria having characteristics which differ from those of previously described species, isolated from more temperate waters. Of particular interest, however, were those bacteria included in clusters numbered 1, 2 and 6. The main characteristics of these clusters are shown in Table 1. Whilst the bacteria of clusters 1 and 2 resembled members of the genera *Vibrio/Aeromonas*, they differ from previously described species in a number of respects, and it is possible that they may represent a group of bacteria with characteristics intermediate between these two genera. The bacteria comprising cluster 6 showed considerable similarity to the Group F organisms described by Furniss *et al.*, (1977). It has recently been proposed by Lee *et al.*, (1981) that this widely distributed group of bacteria should be placed in the genus *Vibrio* and designated *V. fluvialis* sp. nov. Table 2 shows some of the characters proposed by Lee *et al.*, (1981) as useful for the differentiation of *V. fluvialis* from other members of the genus. Whilst a number of differences are evident between the Antarctic vibrios and *V. fluvialis* there is considerable similarity between the two groups. Two biotypes have, however, already been proposed for *V. fluvialis* and it is probable that others may be formed when further taxonomic studies are made of this interesting group of bacteria.

SUMMARY

One hundred and forty four Gram-negative bacteria, indigenous to the maritime Antarctic environment, together with six reference strains, were examined for ninety four unit characters, including morphology, production of exoenzymes, growth temperature characteristics and ability to utilize various organic carbon sources. The resulting data were analysed using the S_{SM} and S_J similarity coefficients with the UPGMA algorithm. At a 70% level of similarity, 97% of the isolates were included in ten clusters. 50% of the isolates were identified as belonging to the genera *Aeromonas/Vibrio*; 22%, *Alcaligenes*; 2%, *Pseudomonas*; and 2% *Flavobacterium*. The remainder could not easily be identifed with bacteria previously described in the Eighth edition of Bergey's Manual of Determinative Bacteriology. Whilst some of these isolates resembled a proposed new species, *Vibrio fluvialis* sp. nov., others may well represent previously undescribed bacteria.

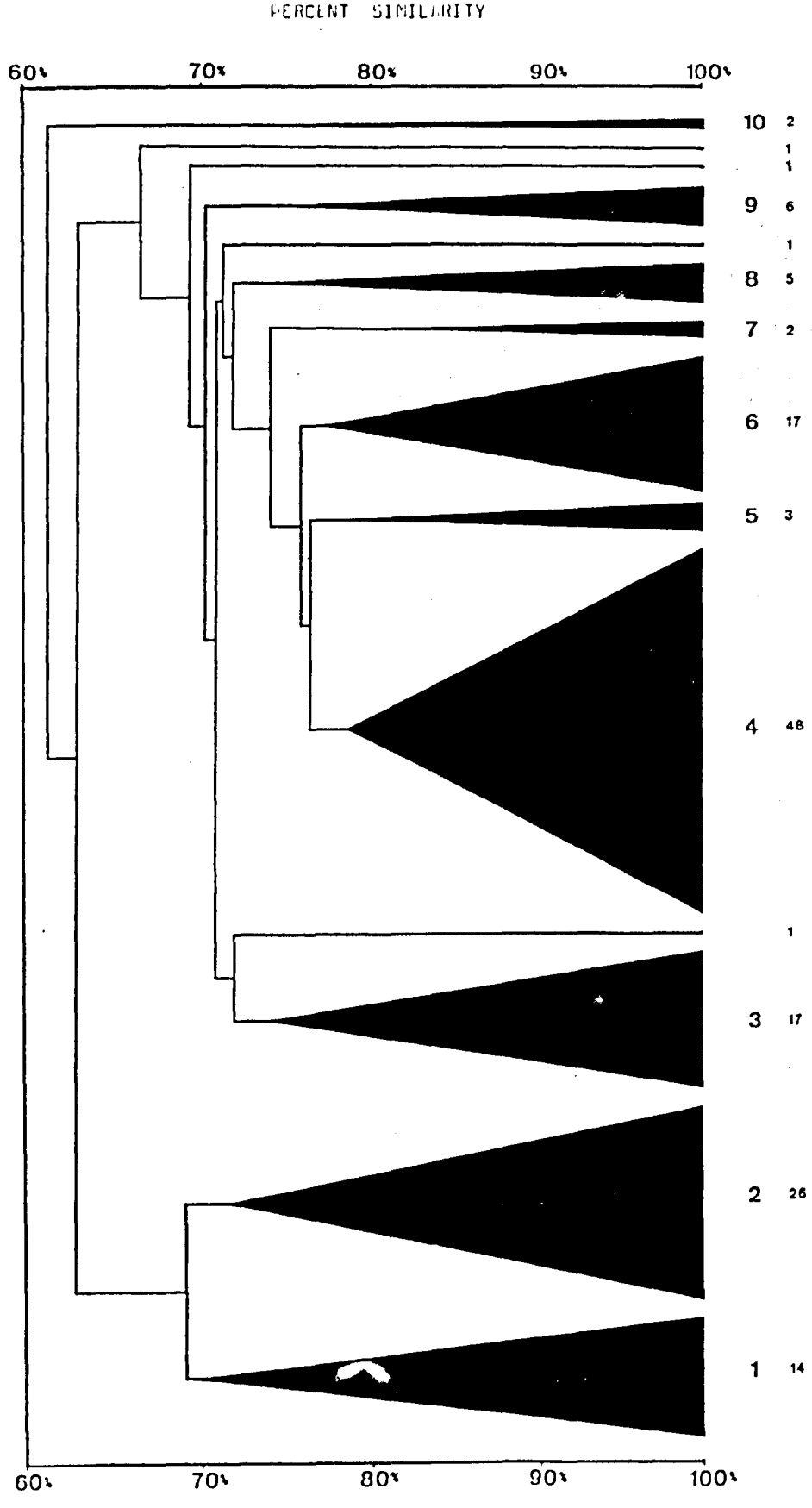

FIGURE 1. Simplified dendrogram showing the relationships between clusters based on the S_{SM} coefficient and UPGMA algorithm.

TABLE 1. MAJOR CHARACTERISTICS OF ISOLATES COMPRISING CLUSTERS 1, 2 AND 6

Character	1	2	6
Motility	+	+	+
Polar flagellum	+	+	+
Asporogenous rods	+	+	+
Gram stain	-	-	-
Oxidase	+	+	+
Fermentation of glucose	+	+	+
Gas from glucose	-	-	-
0/129 sensitivity (10 mcg)	-	-	-
Nitrate reduction	+	+	+
Sucrose utilization	+	+	+
Arginine utilization	+	+	-
Pigment produciton	+	+	+
Psychrophilic/psychrotrophic	+	+	+
Growth without added NaCl	+	+	-

TABLE 2. COMPARISON OF TESTS USED TO CLASSIFY *Vibrio fluvialis* (LEE *et al.*, 1981) AND THE ANTARCTIC ISOLATES IDENTIFIED AS *Vibrio*

	Vibrio fluvialis	Antarctic vibrios
Gram stain	-	-
Motility	+	+
Fermentation of glucose	+	+
Oxidase	+	+
0/129 sensitivity (10 mcg)	-	-
(150 mcg)	+	N.D.
Novobiocin sensitivity	-	-
Lysine	-	-
Ornithine	-	-
Voges-Proskauer	-	-
Indole produciton	-	-
Proprionate	+	(+)
Ethanol	+	(+)
Sucrose	+	+

N.D. = not determined
(+) = less than 50% of isolates posses this character.

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