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A numerical taxonomic study of some pigmented bacteria isolated from Organic Lake, an antarctic hypersaline lake

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Abstract. A study was made of a group of moderately halophilic, heterotrophic, pigmented strains isolated from Organic Lake, Antarctica. These strains were Gram-negative, non-motile, had an aerobic metabolism and a mol% G + C content of their DNA in the range 35–41, indicating that they may be members of the *Flavobacterium-Cytophaga* group. A numerical taxonomic study involving 134 characteristics compared the antarctic strains with reference strains from *Flavobacterium, Cytophaga* and *Flectobacillus*. The antarctic strains formed two clusters that did not contain any reference strains suggesting that they may represent two new species of the genus *Flavobacterium*.

Key words: *Flavobacterium* – *Cytophaga* – Antarctic – Halophile – Hypersaline – Numerical taxonomy

Organic Lake is a hypersaline, meromictic lake of marine origin, located in the Vestfold Hills region of Antarctica. The lake had a maximum depth of 7.5 m in 1984 and was anoxic below 4–5 m. The concentration of salts in the monimolimnion of the lake is approximately six times that of seawater. The biological life in this extreme environment is limited to a microbiota of low species diversity (Franzmann et al. 1987c), and organisms inhabiting such an environment may represent novel taxa. New species of the genus *Halomonas, Halomonas subglaciescola* (Franzmann et al. 1987a), and *Halomonas meridiana* (James et al. 1990) have been isolated from the lake. Pigmented bacteria have been regularly cultured from the lake. A taxonomic study of a collection of these orange and yellow pigmented bacteria is reported here.

Materials and methods

Bacterial strains. The antarctic strains were isolated by allowing single drops of Organic Lake water to flow across the surface of Organic Lake Water agar or cysteine (0.05% w/v) Organic Lake Water agar. Cells from microcolonies on these plates were isolated by micromanipulation. Isolated strains were transferred to Artificial Organic Lake agar (Franzmann et al. 1987a, c). The pigmented strains were routinely maintained on 3% Artificial Organic Lake Peptone (0.5% w/v) agar. The strain numbers, the year of isolation, and the depth of the lake from which they were obtained are given in Table 1.

The reference strains were *Flavobacterium breve* NCTC 11099^T, *Flavobacterium multivorum* UQM 2747 (Hayward and Sly 1984), *Flectobacillus glomeratus* ACAM 111 (McGuire et al. 1987), *Cytophaga lytica* NCIMB 1423^T, *Cytophaga marinoflava* NCIMB 397 (Colwell et al. 1966), *Cytophaga* sp. NCIMB 249 (Hayes 1963), *Flavobacterium* sp. NCIMB 259 (Hayes 1963), *Flexibacter* sp. NCIMB 275 (Hayes 1963), *Halomonas subglaciescola* ACAM 12^T, *Halomonas subglaciescola* ACAM 21, *Halomaonas elongata* ATCC 33173^T, *Halomonas halmophila* NCIMB 1971^T, *Deleya aesta* NCIMB 1980^T, and *Deleya halophila* CCM 3662^T. The reference strains were obtained from the Australian Collection of Antarctic Microorganisms (ACAM), University of Tasmania, Tasmania, Australia; the American Type Culture Collection (ATCC), Rockville, Maryland, USA; the Czechoslovak Collection of Microorganisms (CCM), J.E. Purkyne University, Brno, Czechoslovakia; the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen University Research Company, Aberdeen, Scotland; the National Collection of Type Cultures (NCTC), London, England; and the University of Queensland Department of Microbiology (UQM), St. Lucia, Queensland, Australia.

Numerical taxonomy testing procedures. The basal medium used was 3% Artificial Organic Lake Peptone Broth (3% AOLPB), being Artificial Organic Lake Peptone broth (Franzmann et al. 1987a) modified by the reduction of the NaCl concentration to 3% (w/v). Solid medium was prepared by the addition of 15 g/l agar to 3% AOLPB and the pH adjusted to 7.3 prior to sterilisation. During sterilisation the pH dropped to 7.1. Cultures were incubated for 10 days at 25°C unless specified otherwise, and for an additional 18 days if results were inconclusive because of poor growth. The given salinity and temperature conditions were chosen because they are near optimal for the pigmented antarctic strains. These conditions, however, restricted the choice of reference strains, precluding the inclusion in the study of the non-halotolerant type species of the genus *Flavobacterium, Flavobacterium aquatile* ATCC 11947^T, and the psychrophilic type strain *Flectobacillus glomeratus* ATCC 43844^T.

Morphological characteristics and Gram-stain. Gram stains were done using the method described in Skerman 1967. Cell size was

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Table 1. Strains from Organic Lake used in this study, dates of sample collection and the depth of the lake from which the strains were obtained (Franzmann et al. 1987b).

Strains	Date	Depth (m)
ACAM 1	24 October 1984	3
ACAM 2	24 October 1984	2
ACAM 40, ACAM 41, ACAM 56, ACAM 62	1 May 1986	2
ACAM 43, ACAM 44, ACAM 45, ACAM 46	1 May 1986	5
ACAM 48, ACAM 49	1 May 1986	4
ACAM 51, ACAM 52, ACAM 53, ACAM 54	1 May 1986	3

measured from photographs of microcolonies examined at $\times 1000$ phase contrast using a Laborlux 12 Leitz microscope (Franzmann and Skerman 1981). Cell length was categorised as either ≤ 2 µm, > 2 µm and ≤ 4 µm, > 4 µm and ≤ 6 µm, or > 6 µm. Cell width was categorised as either ≤ 0.4 µm, > 0.4 µm and ≤ 0.8 µm, > 0.8 µm and ≤ 1.2 µm, or > 1.2 µm. Colonies were examined after 5 days growth with a stereo microscope (Nikon) for attributes of size (≤ 1 mm, > 1 mm), colour (white, off-white, orange, yellow), form (circular, irregular, rhizoid), elevation (flat, convex, umbonate), surface type (smooth, wrinkled, striated) and edge type (entire, undulate, filamentous).

Motility. Broth cultures in the early logarithmic phase of growth were examined for motility (by flagella) by the hanging drop method (Skerman 1967). To test for spreading growth and gliding motility strains were grown on basal medium with 1.0 g/l Lab Lemco added and the peptone content reduced to 0.5 g/l. The edges of colonies were examined for cells exhibiting gliding after growth on a thin layer of the low peptone agar. Gliding motility was further tested on the medium of Anacker and Ordal (Anacker and Ordal 1959), modified by the addition of salts and vitamins in the concentrations given for the basal medium.

Biochemical characteristics. Strains grown on basal agar without KNO_3 were tested for catalase (Smibert and Krieg 1981) and for cytochrome oxidase using "Oxidase BR64 Identification Sticks" (Oxoid) and by Kovac's method (Smibert and Krieg 1981). Nitrate reduction (Smibert and Krieg 1981), DNase activity (Jeffries et al. 1957), and phosphatase activity (Smibert and Krieg 1981) were tested. Strains were tested for amyolytic activity (Skerman 1967), caseinolytic activity on basal agar with 10% (w/v) skim milk powder added, agarolytic activity, chitinolytic activity (Skerman 1967), hydrolysis of Tween 20 and Tween 80 (Skerman 1967), and gelatin liquifaction using commercially prepared gelatin-charcoal disks ("Chargels", Oxoid). Phenylalanine deaminase activity, ornithine decarboxylase activity, malonate utilisation, urease activity, esculin hydrolysis and β-galactosidase activity were tested using the Micro ID unit (Organon Teknika Pty. Ltd.), (Vreeland 1980). Inoculum was prepared for the Micro ID tests by suspending cells in 3% (w/v) aqueous NaCl, and the results were read after 24 hours. Colonies were examined for a bathochromic shift in the presence of 20% KOH (Reichenbach et al. 1981).

Physiological characteristics. Strains were tested for growth at 5°C, 0°C and 37°C over a period of 8 weeks; in basal broth adjusted with KOH and HCl to pH 4, 5, 6, 8 and 9; and in basal broth with a NaCl concentration of 0%, 5%, 10%, 15%, and 20%.

Oxygen relations. The strains were tested for oxidative/fermentative metabolism of glucose using a modified version of Hugh and Liefson's medium (Smibert and Krieg 1981). The medium for anaerobic incubation was boiled in a water bath for 5 min and allowed to cool prior to inoculation. Growth under anaerobic conditions (Gas pak, BBL) was tested on basal medium without KNO_3, and on basal medium with 0.2% (w/v) KNO_3 and 1% (w/v) peptone.

Carbohydrate metabolism. Acidic and alkaline reactions of carbohydrate cultures were tested in basal broth with 1% (w/v) filter-sterilised carbohydrate added. After 10 days growth, bromothymol blue indicator was added (Smibert and Krieg 1981). Carbohydrates tested were galactose, fructose, lactose, mannitol, maltose, glucose and arabinose.

Carbon source utilisation. Growth stimulation was tested on basal agar without peptone, 0.01% (w/v) yeast extract, and 0.1% (w/v) carbon source. Stimulation was assessed by visual comparison of colony growth with a control which contained no carbon source. Carbon sources tested were acetate, adonitol, alanine, arabinose, arginine, asparagine, cellobiose, citrate, cysteine, fructose, fumarate, galactose, glucose, gluconic acid, glutamate, glycine, glycerol, histidine, β-hydroxybutyrate, hydroxyproline, inositol, isoleucine, alpha-ketoglutarate, lactate, lactose, leucine, lysine, lysine dihydrochloride, malate, maltose, mannitol, mannose, methionine, ornithine, proline, proprionate, pyruvate, raffinose, alpha-rhamnose, salicin, serine, succinate, sucrose, tartrate, threonine, trehalose, tyrosine, tryptophan, valine and xylose.

Antibiotic susceptibility. Susceptibility to the following antibiotics was tested by the disk method: streptomycin 10 µg, bacitracin 10 units, chloramphenicol 30 µg, tetracycline 30 µg, ampicillin 10 µg, neomycin 30 µg, novobiocin 30 µg, nalidixic acid 30 µg, penicillin G 10 U, polymixin B 300 units, gentamicin 10 µg, vibriostat agent 0/129 10 µg and 0.02% (w/v) $HgCl_2$.

Estimation of testing error. Testing of three strains (ACAM 44, ACAM 54, and *Deleya aesta*) was duplicated. The error estimate was calculated as the percentage of dissimilar results across the duplication.

Numerical analysis. The results were coded in binary format. The data were processed by two methods. The first was by calculation of the measure of similarity between strains as the information statistic and clustering on the basis of information gain as described by Lance and Williams (1967). The second was by calculation of the measure of similarity between strains with the Jaccard coefficient and complete linkage clustering (Sneath and Sokal 1973). Analyses were performed by programs from the Taxon Library Programs (Commonwealth Scientific and Industrial Research Organisation, Division of Computing Research, Canberra, Australia).

Determination of DNA base composition. DNA was extracted and purified by the method of Marmur (1961). The mol% G + C values were determined in 0.1 SSC by the thermal denaturation method, using a Pye Unicam SP8-200 Series UV/Vis Spectrophotometer and a Pye Unicam SPY 876 Series 2 Temperature Program Controller. *Escherichia coli* UQM 1803 was the control and mol% G + C values were calculated from the equation

$$\%G + C_{unknown} = \%G + C_{UQM\ 1803} + 2.08(T_{m\ unknown} - T_{mUQM\ 1803})$$

(Owen and Hill 1979).

Absorption spectra. Strains were grown in 3% AOLPB at 25°C, harvested by centrifugation and the pigments were extracted at room temperature in methanol. Cell debris was removed by centrifugation and the absorption spectra in the 350–600 nm range were recorded with a Varian, Cary 219 Spectrophotometer.

The effect of salt concentration on growth rate. The growth response of ACAM 2 and ACAM 43 was tested in AOLPB with NaCl concentrations adjusted to 0.2, 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 14, 16, 18, 20, 22, 23, 24, and 25% (w/v). The range of total salt concentration was

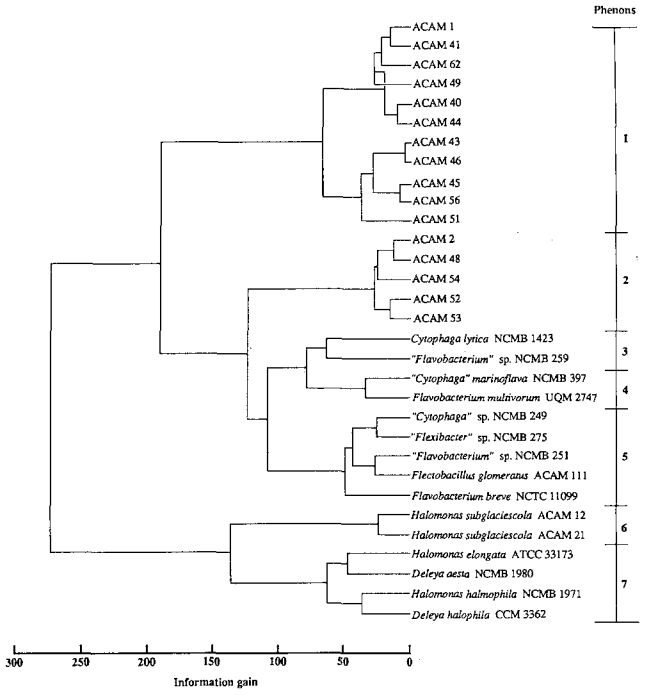

Fig. 1. Dendrogram obtained using the information statistic with clustering on the basis of information gain

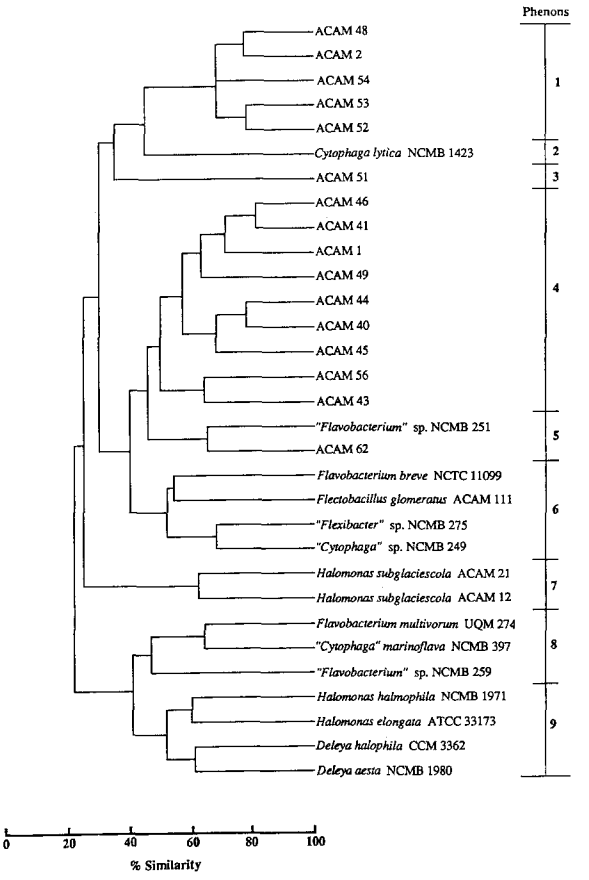

Fig. 2. Dendrogram obtained using the Jaccard coefficient and complete linkage clustering

2.2–27% (w/v). For each strain 0.5 ml of a logarithmically growing broth culture was inoculated into 14.5 ml of broth. The broth were incubated with shaking at 18°C for two weeks. Cell density was measured by absorbance at 540 nm with a Spectronic 20D (Milton Roy Co) at appropriate time intervals. The relative growth rate at each salt concentration was measured as the gradient of a plot of absorbance against time during the logarithmic phase of growth (Ratowsky et al. 1983). The relative growth rates were plotted against NaCl concentration to estimate the optimum NaCl concentration for growth. Absorbance measurements made of a dilution series of turbid cultures of ACAM 2 grown in 1%, 10%, and 18% AOLPB and of ACAM 43 grown in 4%, 10%, and 14% AOLPB indicated a linear relationship existed between absorbance and cell concentration in the experimental range of cell density.

Determination of the theoretical minimal temperature for growth. The theoretical minimal temperature for growth (T_{MIN}) was determined for ACAM 2 and ACAM 43 in AOLPB with the NaCl concentration adjusted to 5% (w/v), using the method of McMeekin and Franzmann (1988) and the T_{MIN} values obtained by the method of Ratowsky et al. (1983).

Results

All the pigmented strains included in this study were Gram-negative, rod-shaped, non-motile (by flagella) cells, positive for growth at pH 5–8, DNase, growth in 5% AOLPB, and phosphatase and all were negative for gas production from nitrate, lysine decarboxylase, malonate utilisation, chitin hydrolysis, anaerobic growth without KNO_3, sensitivity to polymixin B 300 units and gentamicin 10 μg, and growth stimulation by inositol, arabinose, methionine, isoleucine, leucine and valine.

The pigmented strains from Organic Lake did not cluster with the reference strains. They formed two phenons, labelled 1 and 2 in the information statistic dendrogram (Fig. 1), and 1 and 4 in the Jaccard dendrogram (Fig. 2).

The five members of phenon 1 in the Jaccard dendrogram are yellow pigmented. The mol% G+C of the DNA of three members of this group, ACAM 2, 48, and 54 is 40, 41, and 39 respectively. The optimum concentration of NaCl for growth in AOLPB was determiend for ACAM 2 to be between 2 and 4%, or 4 and 6% total salts at 18°C. All of these strains grew at pH 9 and in media containing 15% NaCl or 17% total salts. The cell widths range from 0.5 μm to 0.8 μm and the cell lengths range from 1.2 μm to 11.5 μm. They are aerobic but one strain (ACAM 54) was able to grow anaerobically with KNO_3. Colonies are yellow, circular, entire and convex. All members of this group are positive for catalase, cytochrome oxidase, Kovacs oxidase, nitrate reduction to nitrite, starch hydrolysis, esculin hydrolysis, β-galactosidase, gelatin liquefaction, lipolysis of Tween 20, growth stimulation by arginine, gluconic acid, pyruvate, maltose, ornithine and lactose, and production of acid from arabinose, maltose and mannitol. They are sensitive to novobiocin 30 μg, chloramphenicol 30 μg, ampicillin 10 μg, penicillin G 10U, erythromycin 15 μg and cephalothin 30 μg and are resistant to 0/129 10 μg and neomycin 30 μg. They were all negative for ornithine decarboxylase, spreading growth on low peptone agar, agar hydrolysis, casein hydrolysis, and growth stimulation by adonitol, rhamnose, salicin, glycine, hydroxyproline, mannitol, xy-

Table 2. Characteristics which differ between the strains comprising phenon 1 (Fig. 1) and *Cytophaga lytica* NCIMB 1423[T]

Characteristic	Phenon 1	*Cytophaga lytica*
Growth at pH 9	+	−
Nitrate reduction to nitrite	+	−
Novobiocin 30 μg	S[a]	R[a]
Chloramphenicol 30 μg	S	R
Penicillin G 10 units	S	R
Growth on 0% NaCl	+	−
Growth on 15% NaCl	+	−
Agarolytic	−	+
Flat colony	−	+
Convex colony	+	−
Spreading growth	−	+
Growth stimulation on:		
Glycine	−	+
Hydroxyproline	−	+
Lysine	−	+
Histidine	−	+

[a] S = sensitive, R = resistant

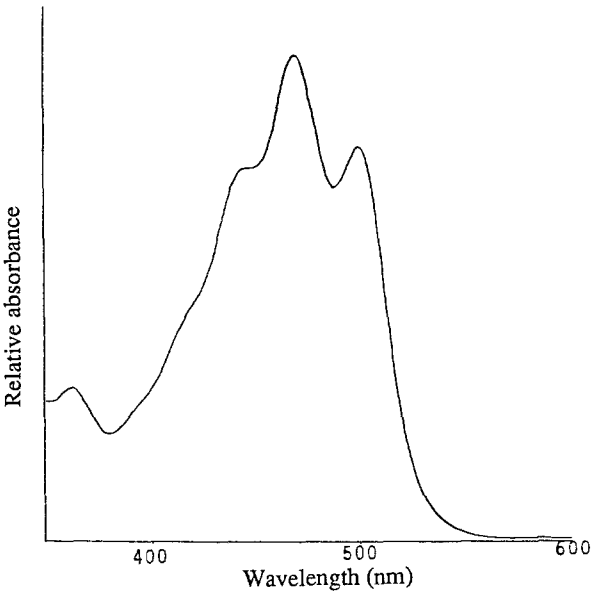

Fig. 3. Absorbance spectrum in methanol of the whole pigment extract of ACAM 1, a strain from phenon 1 (Fig. 1)

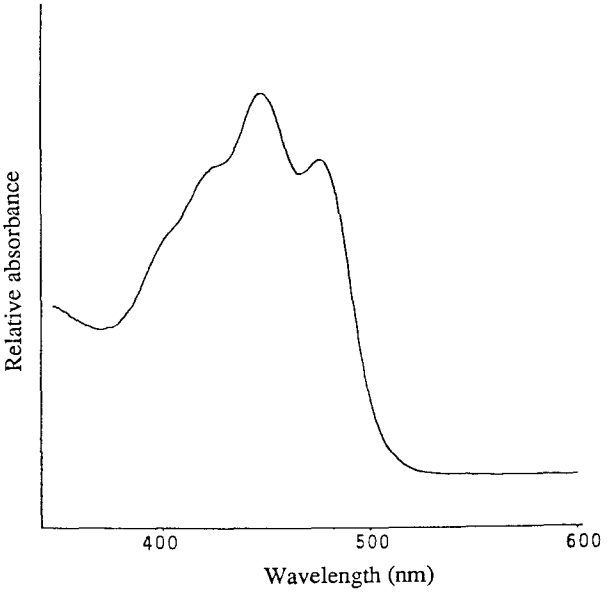

Fig. 4. Absorbance spectrum in methanol of the whole pigment extract of ACAM 54, a strain from phenon 2 (Fig. 1)

lose, serine, trehalose, asparagine, lysine, histidine, arabinose, cysteine, threonine, tyrosine, glycerol, and maltose.

Of the reference strains included in this study, the members of phenon 1 appear to be most closely related to *Cytophaga lytica* (Fig. 2), however, as several characteristics differ between *Cytophaga lytica* and the members of phenon 1 (Table 2) it is unlikely that they are members of the same species.

Phenon 1 of the information statistic dendrogram (Fig. 1) and phenon 4 of the Jaccard dendrogram (Fig. 2) contain the second group of antarctic strains. The membership of these two phenons differs in that ACAM 51 and 62 fall outside of phenon 4 of the Jaccard dendrogram. The level of similarity amongst the members of these phenons is less than that of the first group of antarctic organisms described. It is probable that this arose in part from difficulties that occurred with testing these strains, which often grew poorly and sometimes not at all. DNA-DNA hybridisation studies should be performed to determine the degree to which the observed heterogeneity, in this group, is genetically based.

All the members of phenon 1 (Fig. 1) are orange pigmented. The mol% G + C of the DNA of three members of this group, ACAM 43, 44 and 56 is 35, 39 and 36 respectively. The optimum concentration of NaCl for growth in AOLPB was determined for ACAM 43 to be between 2 and 4%, or 4 and 6% total salts. All strains grew in AOLPB containing 10% NaCl or 12% total salts. Cell widths were in the range 0.4 μm − 0.8 μm and cell lengths in the range 1.7 μm − 11.7 μm. Colonies were orange, circular, entire and convex. The members of this group were all positive for cytochrome oxidase, esculin hydrolysis, starch hydrolysis, sensitivity to novobiocin 30 μg, chloramphenicol 30 μg and erythromycin 15 μg and resitant to 0/129 10 μg. They were all negative for reduction of nitrate to nitrite, ornithine decarboxylase, β-galactosidase, growth in 20% NaCl, growth at 37° C, spreading growth on low peptone agar, agar hydrolysis, casein hydrolysis, acid production from galactose and growth stimulation by proline, sucrose, rhamnose, salicin, glucose, galactose, glycine, hydroxyproline, mannose, gluconic acid, maltose, serine, trehalose, tryptophan, alanine, cysteine, histidine, arabinose, tyrosine and glycerol.

Absorption spectra of pigments extracted from representative strains from phenons 1 and 2 (Fig. 1) are presented in Figs. 3 and 4. These spectra are typical of carotenoids. Flexirubin pigments are characteristic for many terrestrial members of the *Flavobacterium-Cytophaga* group, but have been rarely found in organisms of this type from marine environments (Reichenbach et al. 1981). The Organic Lake strains did not exhibit a

bathochromic shift on the addition of alkali indicating that they did not contain flexirubin pigments.

The theoretical minimum temperatures for growth of ACAM 2 and ACAM 43 were 268 K and 267 K respectively, placing these strains in the range for psychrotrophic organisms (McMeekin et al. 1988). The estimate of error for the numerical taxonomy testing was 4.2%.

Discussion

Gram-negative, aerobic, non-motile, pigmented bacteria are routinely isolated from marine environments (Hayes 1963; Simudu et al. 1986) and have been assigned to the genera *Flavobacterium* and *Cytophaga*, which are recognised as being closely related (Reichenbach and Weeks 1981). These two genera are distinguished on the basis that members of the genus *Cytophaga* exhibit gliding motility, and members of the genus *Flavobacterium* do not. The utility of defining two separate genera on the basis of a single phenotypic characteristic, gliding motility, for a group of organisms which are similar on the basis of a number of chemosystematic and genetic characteristics has been questioned (Holmes et al. 1984a; Shewan and McMeekin 1983; Reichenbach and Weeks 1981). A recent analysis of 16S rRNA sequences suggests that gliding motility is an ancestral property of the phylum to which the genera *Cytophaga* and *Flavobacterium* belong, and that gliding and non-gliding strains may be closely related (Woese et al. 1990). Consequently it may no longer be appropriate to define *Flavobacterium* by a lack of gliding motility. The flavobacteria-bacteroides phylum also contains members of the genera *Flexibacter* and *Flectobacillus*. The delineation and description of genera within this phylum has yet to be resolved, and will require the establishment of groups based on phylogenetic relationships amongst a large number of the described species, and the elucidation of suitable phenotypic characteristics to describe those groups.

None of the seven species of the genus *Flavobacterium* listed in *Bergey's Manual of Systematic Bacteriology* (Holmes et al. 1984a) were isolated from marine or hypersaline environments. Recent taxonomic studies of this group have largely focussed on isolates from the clinical environment (Reichenbach and Weeks 1981; Holmes et al. 1983, 1984b, 1988). The group of reference strains NCIMB 249, NCIMB 251, NCIMB 259 and NCIMB 275, in this study, were drawn from an earlier study of marine flavobacteria (Hayes 1963); *Cytophaga marinoflava* (Colwell et al. 1966) is a non-gliding marine strain, originally assigned to the genus *Cytophaga* because of the heterogeneity which existed within the genus *Flavobacterium* at the time of its description; and *Flectobacillus glomeratus* was isolated from an antarctic lake of marine salinity (McGuire et al. 1987).

Interest in the moderately halophilic bacterial flora of hypersaline environments has followed investigation of the extreme halophiles, belonging to the kingdom Archaea, isolated from these environments. Similarity has been found in the composition of the bacterial flora between hypersaline environments and their non-hypersaline counterparts (Rodriguez-Valera 1988). Bacteria with a phenotype characteristic of members of the genus *Flavobacterium* have been isolated from salterns and hypersaline soils, however, a high mol% G + C content of their DNA precluded their inclusion in this genus (Quesada et al. 1983, 1987). A comparative phenotypic study of moderately halophilic flavobacteria is limited by the non-availability of moderately halophilic reference strains. The range of reference strains is limited to marine and halotolerant organisms. The selection of a single set of conditions optimal for the strains included in this study which range from halotolerant to moderately halophilic was not possible.

Salt concentration is known to affect the expression of many characters including antibiotic sensitivity (Merkel 1972; Hebert and Vreeland 1987), motility (Rosenburg 1983), urease activity, reduction of nitrate, and production of acid from carbohydrates (Hebert and Vreeland 1987). In general it is not a good practice to amalgamate phenotypic data from different laboratories when undertaking a numerical taxonomy, as the error level is unacceptable (Sneath and Sokal 1973). Even more so in this case it is not useful to compare the phenotype of the antarctic strains with those of the members of *Flavobacterium* not included in this study, as the latter phenotypes were observed under different cultural conditions i.e. minimal salinity. To circumvent these problems and gain a clearer picture of the relationship of the halophilic antarctic strains and existing flavobacterial species a genetic study is currently in progress. In conclusion, it appears that the pigmented strains from Organic Lake belong to the genus *Flavobacterium*, as it is currently defined, and may represent new species.

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