Numerical Taxonomy of Aquatic *Acinetobacter* Isolates

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SUMMARY

Two hundred and seventy Gram-negative strains, representing aquatic members of the genus *Acinetobacter*, were isolated and compared with 48 related clinical isolates and reference strains from a variety of genera. For each isolate, a total of 96 coded characters derived from 89 characteristics was determined using morphological, physiological, nutritional and biochemical features, in addition to sensitivities to several antibiotics and inhibitory agents. The data were analysed by computer to obtain a simple matching coefficient for each pair of strains. Clustering was performed by the unweighted pair-group method of association.

Two major phenons were formed which excluded the oxidase-positive, motile or facultatively anaerobic strains. Within each phenon, three 'subphenons' were delimited. The two phenons, comprising 291 isolates, were tentatively differentiated at the species level, while their shared characteristics indicated that both phenons should be included in the genus *Acinetobacter*. Phenon 2 contained most of the clinical isolates and corresponded to the type species *Aci. calcoaceticus* as described originally by Baumann, Doudoroff & Stanier (1968). Phenon 1 was composed almost entirely of aquatic isolates and may prove to represent a second species of a less biochemically-active nature.

Distinguishing characters have been suggested as diagnostic criteria for the differentiation of these two phenons.

INTRODUCTION

Organisms corresponding to the general description of Gram-negative, aerobic, non-motile cocci or coccobacilli have been classified by various authors as *Diplococcus*, *Neisseria*, *Mima*, *Herellea*, *Moraxella*, *Bacterium*, *Achromobacter*, *Alcaligenes* and, most recently, *Acinetobacter*. [See Henriksen (1973) for an extensive literature review.] Although their potential for pathogenicity appears to be low, such bacteria are evidently opportunistic, capable of causing severe and even fatal infections when resistance is lowered. Most reported isolations have been of human origin, including skin (Brodie & Henderson, 1964), saliva (Shklair, 1965), urine (Rosebury, 1962), cerebral spinal fluid (Billing, 1955) and the genito-urinary system (Samuels *et al.*, 1972). Some of the major diseases in which such bacteria have been implicated include meningitis (Olafsson, Lee & Abernathy, 1958; Burrows & King, 1966; Taindel, Oproiu & Burlacu, 1971), 'therapy-resistant gonorrhoea' (Samuels *et al.*, 1972), brain abscesses (Opsahl, 1961), vaginal infections (Langhof & Franken, 1964) and ear infections (Goldberg, Ronis & Ronis, 1966). In many cases, infections occurred after such predisposing factors as extensive antibiotic treatment, malignancies (Henriksen, 1973), immunosuppression, or use of respirators (Reinarz, 1965).

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Isolations have also been reported from soil and water (Baumann, 1968; Warskow & Juni, 1972). Bennett (1969) reported that *Acinetobacter* spp. constituted 21 % of the heterotrophic bacterial population in Lake Ontario and Lake Superior. Seyfried (1973) observed that the *Acinetobacter* density had increased to 39 % in Lake Ontario. A high level of correlation ($P < 0.001$) between the incidence of this genus and the level of faecal pollution in Lake Ontario was noted by both workers.

Much of the literature concerning this group of organisms is difficult to interpret due to confusion over its nomenclature and lack of a widely accepted classification scheme. In 1962 the International Committee on Nomenclature of Bacteria (ICNB, now known as International Committee on Systematic Bacteriology, ICSB) appointed an *ad hoc* committee to study the taxonomic status and nomenclature of the genus *Moraxella*, the species *Bacterium anitratum*, the tribe Mimeae and other apparently related organisms. Four years later this *ad hoc* committee was designated the Subcommittee on *Moraxella* and Allied Bacteria. In 1971 the ICSB (Judicial Commission, 1971) decided to include all oxidase-negative *Moraxella* in the genus *Acinetobacter* with only one species, *calcoaceticus* (Beijerinck, 1911). This genus and the remaining members of the genus *Moraxella* were temporarily assigned to the family Neisseriaceae for practical purposes. Since then, extensive transformation studies (Juni, 1972; Brooks & Sodeman, 1974) have shown little, if any, relationship between *Acinetobacter* and the oxidase-positive *Moraxella* and *Neisseria*.

In view of the lack of research on aquatic strains of *Acinetobacter*, their possible role in water pollution and the unsatisfactory nature of the present classification system, further studies seemed appropriate. Our aim was to characterize and compare a collection of acinetobacters, isolated from various bodies of water in southern Ontario, with related clinical and reference strains by use of numerical taxonomy. From the results obtained, we hoped to formulate a workable diagnostic key which would take into account the aquatic strains which do not conform well to the existing system of classification.

METHODS

Strains. Twenty-two sites were sampled from rivers and lakes within a 100-mile radius of Toronto and samples were plated using membrane filtration technique. Two hundred and seventy Gram-negative, non-motile, aerobic cocci or coccobacilli were isolated from plates of Trypticase Soy Agar (TSA; BBL): these were all designated by numbers with PS prefixes. A single strain isolated from hen manure was also included in this study.

Clinical isolates, detailed in Table 1, were obtained mainly from the Ontario Public Health Laboratory at Toronto.

Twenty-one reference strains from culture collections (Table 2) were included as 'marker strains' for the interpretation of clusters derived from the computer analysis.

Duplicate cultures of each isolate were maintained at 4 °C on TSA slants in screw-capped tubes and subcultured every 2 months. Unless otherwise specified, all tests (listed in Table 3) were inoculated by loop from an 18 h TSA culture and incubated at 25 °C. Results were recorded after 24 and 48 h, and again after one week.

Morphological studies. Cellular morphology and staining characteristics were determined using Hucker's modification of the Gram stain (*Manual of Microbiological Methods*, 1957) on 24 h TSA cultures. Duguid's wet India ink method (1951) was employed to demonstrate capsule formation in 24 h TSA cultures. Motility was tested using a twice-transferred 18 h Trypticase Soy Broth (TSB; BBL) culture which was stab-inoculated into Motility Test Medium (Difco). Observation of motility was facilitated by incorporating 0.005 %

Table 1. *Strains from clinical specimens*

Designation as received	Source†	Specimen	No. of strains
<i>Acinetobacter anitratum</i> *	MHO	Urine	3
		Vagina	1
		Mouth	1
		Throat	3
		Hand	1
		Unspecified	5
<i>Bacterium anitratum</i>	MG	Unspecified	1
<i>Mima polymorpha</i> *	MHO	Unspecified	9
<i>Mima polymorpha</i> var. <i>oxidans</i>	MHO	Unspecified	1
<i>Neisseria catarrhalis</i>	HSC	Unspecified	1
			Total: 26

* Present classification would be *Acinetobacter calcoaceticus*.

† MHO, Ministry of Health of Ontario, Laboratory Services Branch, Toronto, Canada. MG, McGill University, Montreal, Canada. HSC, Hospital for Sick Children, Toronto, Canada.

triphenyltetrazolium chloride (Kun & Abood, 1949). Doubtful results were checked in a hanging drop preparation from a similarly grown culture.

Colonial characteristics which proved to be reproducible (consistency and pigment) were recorded after incubation for 48 h on TSA.

Physiological studies. All strains were tested for the ability to grow at 37 °C on TSA after one week of incubation. The effect of initial pH was observed using TSA slants adjusted to pH 5.1, 5.6, 11.0, 11.6 and 12.2 (Rhodes, 1959). Sodium chloride was incorporated into TSA at concentrations (% w/v) of 2.0, 3.0, 3.5, 4.0 and 5.0 in order to assess salt tolerance. The effects of initial pH and of NaCl concentration were recorded after 48 h and again after one week. Anaerobiosis was obtained using jars with Gas-Paks (BBL), and growth on TSA was observed after 48 h under these conditions.

Antibiotic sensitivity testing. Sensitivity to the antibiotics listed in Table 3 was measured with the aid of a multiple inoculating machine (Bio. Tech. Co., Lynden, Ontario, Canada) following the 'Routine Sensitivity Method' of the Hospital for Sick Children, Toronto. Seed cultures were incubated for 4 h in TSB before being used to inoculate two sets of master plates. Four control cultures with known antibiograms were included with each run.

Resistance to inhibitory agents. The effect of sodium lauryl sulphate on growth was determined by incorporating the detergent at a concentration of 0.1 % (w/v) into TSA. Tolerance to KCN was tested with the medium of Rogers & Taylor (1961) containing 0.005 % triphenyltetrazolium chloride.

Biochemical studies. *p*-Aminodimethylaniline oxalate (Difco; 1 % w/v) was used to test for oxidase activity in 24 h TSA cultures. Urease activity was observed on Christensen (1946) Urea Agar (Difco) after 48 h incubation. Reduction of nitrate to nitrite was tested in (Difco) Bacto-Nitrate Broth after 12 and 24 h as described in the *Manual of Microbiological Methods* (1957). To determine ability to liquefy gelatin, a 3 % (w/v) nutrient gelatin plate was stab-streaked, incubated for 48 h and then flooded with 20 % (w/v) sulphosalicylic acid to precipitate the gelatin (Chapman, 1952). Reactions on egg yolk agar were examined on the egg yolk medium of McClung, Heidenreich & Toabe (1946). Well-isolated colonies on Eosin Methylene Blue Agar (EMB; Difco) were examined after 24 and 48 h incubation. The

Table 2. *Strains from culture collections*

Catalogue designation	Designation(s) from other collections	Originally deposited as:
<i>Achromobacter</i> sp. NCIB8250	<i>Vibrio</i> sp. ATCC11171 Baumann <i>et al.</i> (1968) strain 94	' <i>Vibrio</i> 01'
<i>Achr. anitratum</i> NCIB9212	<i>Aci. calcoaceticus</i> ATCC19187 Sneath* strain D411 Schaub & Hauber (1948) strain 93 Hugh & Reese (1967) strain RH2317	<i>Bacterium anitratum</i>
<i>Achr. anitratum</i> NCIB9213	<i>Aci. calcoaceticus</i> ATCC19569 Sneath* strain D410 Hugh & Reese (1967) strain RH2311	<i>B. anitratum</i>
<i>Achr. anitratum</i> NCIB9214	<i>Aci. calcoaceticus</i> ATCC19568 Sneath* strain D409 Hugh & Reese (1967) strain RH2315	<i>B. anitratum</i>
<i>Achr. anitratum</i> NCIB9301	Ferguson & Roberts (1950) strain sw99	<i>Achr. anitratum</i>
<i>Achr. anitratum</i> NCIB9305		<i>Aci. anitratum</i>
<i>Achr. lwoffii</i> NCIB9020		<i>Aci. lwoffii</i>
<i>Achr. lwoffii</i> NCIB9303		<i>Aci. lwoffii</i>
<i>Achr. parvulus</i> ATCC4335		<i>Bacterium parvulum</i>
<i>Achr. venenosum</i> NCIB9022		
<i>Acinetobacter calcoaceticus</i> ATCC15308	<i>Aci. anitratus</i> NCTC7844 Hugh & Reese (1967) strain RH2197	
<i>Aci. calcoaceticus</i> ATCC17903	<i>Aci. anitratus</i> NCTC8102 Hugh & Reese (1967) strain RH2210	B5W
<i>Aci. calcoaceticus</i> ATCC17924	<i>Achr. anitrata</i> NCIB9019 Hugh & Reese (1967) strain RH2380	<i>Aci. anitratum</i>
<i>Aci. calcoaceticus</i> ATCC17925		<i>Aci. lwoffii</i>
<i>Aci. calcoaceticus</i> ATCC19606	Hugh & Reese (1967) strain RH2208	<i>B. anitratum</i>
<i>Aci. lwoffii</i> ATCC15309 (as received, Oct. 1972)	<i>Aci. lwoffii</i> NCTC5866 Pintér & Bende (1967) strain 29	<i>Moraxella lwoffii</i>
<i>Alcaligenes faecalis</i> NCIB8156	<i>Alc. faecalis</i> ATCC8750 Thornley (1960) strain 16	<i>Alc. faecalis</i>
<i>Alc. faecalis</i> SH9220†		
<i>Alc. viscolactis</i> NCIB8596	Thornley (1967) strain 28	<i>Alcaligenes viscosus</i>
<i>Moraxella bovis</i> NCTC8561	<i>Mor. bovis</i> ATCC17947 <i>Mor. bovis</i> CDC KC746 Baumann (1968) strain L-10	<i>Moraxella duplex</i> subsp. <i>bovis</i>
<i>Pseudomonas alcaligenes</i> NCTC8769	Thornley (1967) strain 22	

ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; NCIB, National Collection of Industrial Bacteria, Aberdeen; NCTC, National Collection of Type Cultures, Colindale, London; CDC, Centre for Disease Control, Atlanta, Georgia, U.S.A.

* See Hugh & Reese (1968).

† SH9220, Lyophilized culture from School of Hygiene, Department of Microbiology, University of Toronto.

occurrence of blue colonies and their colour intensity were noted. Ability to grow and growth characteristics on MacConkey Agar (Difco) were observed after 24 and 96 h. Type of haemolysis on sheep blood agar (5 %, v/v, in Difco Heart Infusion Agar) was noted after incubation for 24 and 48 h. Strains were tested for their ability to cause pH change in Malonate Broth (Difco) after incubation for one week. Alkalinization of Simmons (1926) Citrate Agar (Difco) was recorded after 48 h. Methyl red and Voges-Proskauer tests were

Table 3. *Summary of bacteriological tests used in the numerical analysis*

Test	Character states used for coding
Morphological studies	
Shape	Coccus Coccobacillus Rod
Motility	+ or -
Capsule	+ or -
Consistency	Butyrous Mucoid Viscid Dry
Pigment	Cream colonies + diffusible brown pigment Cream Other
Growth in broth	Mucoid sediment Flaky sediment Other (ring, pellicle etc.)
Physiological studies	
Growth at 37 °C	+ or -
Growth at initial pH of	
5.1	+ or -
5.6	+ or -
11.0	+ or -
11.6	+ or -
12.2	+ or -
Growth in presence of NaCl	
2.0 % (w/v)	+ or -
3.0 % (w/v)	+ or -
3.5 % (w/v)	+ or -
4.0 % (w/v)	+ or -
5.0 % (w/v)	+ or -
Anaerobiosis	+ or -
Antibiotic sensitivity testing	
Penicillin G 5 i.u. ml ⁻¹	Resistant or Sensitive (R or S)
Chloramphenicol 16 µg ml ⁻¹	R or S
Cephaloridine 1 µg ml ⁻¹	R or S
Kanamycin 8 µg ml ⁻¹	R or S
Tetracycline 4 µg ml ⁻¹	R or S
Gentamycin 4 µg ml ⁻¹	R or S
Nalidixic acid 12 µg ml ⁻¹	R or S
Ampicillin 16 µg ml ⁻¹	R or S
Resistance to inhibitory agents	
Growth in presence of 0.1 % sodium lauryl sulphate	+ or -
Growth in presence of KCN	+ or -
Biochemical studies	
Oxidase activity	+ or -
Urease activity	+ or -
Nitrate reduction	+ or -
Gelatin liquefaction	+ or -
Reaction on egg yolk	Pearly layer + precipitate Opalescence No reaction

Table 3. (cont.)

Test		Character states used for coding
EMB		Navy colonies Pale blue colonies No growth
MacConkey		Growth + red precipitate Growth No growth
Haemolysis		Complete Greening Darkening None
Malonate		Final pH 7.3 to 7.6 Final pH 7.8 to 9.0 No growth
Simmons (1926) test for citrate utilization		Alkalinization pH unchanged
Methyl red		+ or —
Voges-Proskauer		+ or —
Indole production		+ or —
Utilization of organic compounds as sole sources of carbon (% w/v)		
L-Arabinose	(0.5)	+ or —
D-Glucose	(0.5)	+ or —
Lactose	(0.5)	+ or —
n-Butanol	(0.4)	+ or —
Glycerol	(0.4)	+ or —
Phenol	(0.025)	+ or —
Acetate	(0.4)	+ or —
n-Butyric acid	(0.4)	+ or —
Succinate	(0.4)	+ or —
Fumarate	(0.4)	+ or —
dl-Malic acid	(0.4)	+ or —
Pyruvate	(0.4)	+ or —
n-Butyl-p-hydroxybenzoate	(0.4)	+ or —
Gluconate	(0.5)	+ or —
dl-Aspartic acid	(0.4)	+ or —
L-Tyrosine	(0.4)	+ or —
L-Proline	(0.4)	Alkalinization Acid production No utilization
Acid production from carbohydrates		
Arabinose		Strong Moderate None
Glucose		Strong Moderate None
Lactose		+ or —
Sucrose		+ or —

performed with the M.R.-V.P. medium described in the *Manual of Microbiological Methods* (1957). Indole production was measured in peptone water (Difco), after incubating for 48 h, by the addition of Kovác's reagent.

Utilization of organic compounds as sole sources of carbon. The basal medium of Ayers, Rupp & Johnson (1919) with $\text{NH}_4\text{H}_2\text{PO}_4$ as nitrogen source was employed to investigate growth with 17 organic compounds (Table 3). The concentrations chosen were judged to be low enough to avoid the problem of false negative results due to toxicity, or false positive results caused by the presence of utilizable organic contaminants. Phenol was incorporated at 0.025 % (w/v) to avoid the toxic effects noted by Stanier, Palleroni & Doudoroff (1966) at concentrations of 0.05 % and above. The basal medium was prepared as suggested in the *Manual of Microbiological Methods* (1957) with added agar (1.5 %, w/v) and phenol red (1.8 ml per litre of a 1.0 %, w/v, aqueous solution). The pH was adjusted to 7.2 with NaOH. Arabinose, glucose, lactose and gluconate were sterilized separately by Seitz-filtration and then added to the autoclaved basal medium. Plates containing 25 ml medium were inoculated using the replica plating method of Lederberg & Lederberg (1952) allowing 10 strains per plate. In this way, it was hoped that problems such as substrate exhaustion due to overcrowding, effects of bacterial toxins or enzymes, or starvation of slower growing colonies could be avoided. The master plates of TSA were incubated for 18 h. At the end of each test series, a TSA plate was replica plated to check for successful growth and the presence of any contaminants. Growth and pH changes were recorded after 24 and 48 h.

Scoring of growth was done visually and was judged negative if growth was no greater than on a control plate of basal medium with no added carbon source, or positive if the growth was significantly greater than on the control plate. Doubtful results were checked by inoculating singly on fresh plates, where growth could not be influenced by possible effects of other strains. A repeatedly doubtful result was considered negative.

Acid production from carbohydrates. Production of acid from four carbohydrates was tested using the medium of Board & Holding (1960). The carbohydrates were sterilized separately by Seitz-filtration and added at a concentration of 0.3 %. The basal medium chosen contained no peptone so false negative results due to ammonia production were avoided. The semi-soft agar was stab-inoculated and incubated for 6 weeks. Observations of acid production were made at weekly intervals and a control tube with no added carbohydrate was included to confirm growth and absence of pH change.

Acid production was also tested in a single peptone-containing medium. Triple Sugar Iron agar (TSI; Difco) was selected for this purpose and pH change was recorded after 24 h incubation.

Determination of base ratios. To provide a check on the clusterings obtained by computer analysis, four representative aquatic acinetobacters were chosen for base ratio determinations. A reference strain (*Escherichia coli* 0127:B8 4173 from the Public Health Laboratory, Toronto) with a molar percentage guanine plus cytosine (% G + C) of 50.5 was included. DNA was isolated using the standard method of Marmur (1961) and its concentration measured by Stumpf's (1952) method using calf thymus DNA at $1000 \mu\text{g ml}^{-1}$ as the standard. The % G + C was determined for each strain from its denaturation temperature (T_m) according to the method of Schildkraut & Lifson (1965).

Computation. The package program NT-SYS from the State University of New York (Stoney Brook, New York, U.S.A.) was chosen for the computation. It consisted of a numerical taxonomy system of multivariate statistical programs written by F. James Rohlf and John Kishpaugh of the Department of Ecology and Evolution, and David Kirk of the Computation Centre. Computation was carried out on an IBM S/370-165 electronic

computer at the University of Toronto. The input data consisted of 318 strains by 96 unit characters in a rectangular matrix.

Coding of data. Many characteristics were of the presence/absence type and were simply coded by the two-state method. Properties existing in several quantitatively different states, such as fermentation of arabinose, were coded as quantitative multistate characters (Sneath & Sokal, 1973). The qualitative multistate coding method (Sneath & Sokal, 1973) was employed for characteristics, such as colonial morphology, having several qualitatively different states. The symbol NC (no comparison) was used to replace missing or inapplicable data.

Tests whose results were found not to be reproducible or useful, e.g. all strains were Gram-negative, were omitted from the analysis.

The association coefficient chosen was the simple matching coefficient (S_{sm}) of Sokal & Michener (1958), allowing the inclusion of negative matches.

Strains were clustered by the unweighted pair-group method of association (Sokal & Michener, 1958) and the data output presented as a phenogram.

RESULTS

Arrangement of strains into phenons

The results are represented diagrammatically in the phenogram in Fig. 1. At the 70 % similarity level all but 27 strains were grouped into two major clusters designated phenons 1 and 2. The first phenon encompassed 87 strains and the second 204.

Comparison of phenons 1 and 2

The 291 isolates in these two clusters were considered true acinetobacters as they shared the characteristics necessary for inclusion in this genus (Henriksen, 1973), i.e. Gram-negative with a tendency to resist decolorization; coccoid to coccobacillary cellular morphology; no oxidase activity; non-motile; and strict aerobiosis. Other features common to at least 95 % of the strains are listed in Table 4.

Although the two phenons shared many properties, salient differences responsible for the sharp division above the 71 % similarity level were found. Members of phenon 1 generally had larger coccobacillary cells, approximately 0.7 to 0.9 μm by 0.9 to 1.2 μm , and were usually arranged in pairs. In contrast, most strains belonging to phenon 2 had a smaller cell size with morphology ranging from coccoid, with a diameter of 0.6 μm , to coccobacillary, 0.7 by 0.9 μm , generally arranged singly. Over half of the isolates in phenon 1 produced a diffusible light brown pigment after 24 h of growth on TSA. The tests in which the majority of phenon 2 strains gave positive results, while most of the phenon 1 strains gave negative results, included growth at 37 °C, growth at an initial pH of 11.6, tolerance to 3.0 % (w/v) NaCl, fermentation of glucose and arabinose, and alkalization of Simmons Citrate Agar.

Much wider nutritional spectra were observed among strains belonging to phenon 2. In every case, a higher proportion of phenon 2 isolates could utilize each of the 17 carbon sources tested. In particular, over 75 % of phenon 2 strains could utilize n-butanol, acetate, n-butyric acid and/or proline, whereas less than 30 % of phenon 1 strains could utilize any of these compounds. Many of the physiological and biochemical differences reflected the distribution of the isolates according to their source: 21 of the 26 clinical isolates were included in phenon 2 while the first phenon was almost entirely aquatic in origin, containing none of the clinical isolates.

Table 5 lists the % G + C ratios obtained for four aquatic isolates, in addition to results

Table 4. *Characteristics shared by phenon 1 and phenon 2 strains*

Characteristic	Result*
Motility	—
Cream-coloured colonies 1 to 2 mm diam. on TSA	+
Growth at initial pH	
5.1	—
5.6	+ (92 %)
11.0	+ (91 %)
12.2	—
Growth in presence of NaCl	
2.0 % (w/v)	+ (90 %)
5.0 % (w/v)	—
Anaerobiosis	—
Antibiotics	
Penicillin G	R (84 %)
Kanamycin	S
Tetracycline	S
Gentamycin	S
Nalidixic acid	S
Growth in presence of KCN	+
Oxidase activity	—
Nitrate reduction	—
Blue or navy pigmented colonies on EMB	+ (88 %)
Methyl red	—
Voges-Proskauer	—
Indole production	—
Utilization as sole carbon source	
Lactose	—
Glycerol	—
<i>dl</i> -Malic acid	+ (86 %)
Pyruvate	+ (85 %)
<i>n</i> -Butyl- <i>p</i> -hydroxybenzoate	—
Gluconate	—
Acid production	
Lactose	—
Sucrose	—

* Results listed refer to ≥ 95 % of strains, unless otherwise indicated.

for several reference strains as determined by other workers. The ratios ranged from 41 to 46 % for phenons 1 and 2, but were much higher for two of the ungrouped strains. Phenon 1 strains appeared to have slightly higher % G + C ratios than phenon 2 but more base ratio determinations would be needed to establish this.

A definite separation between the two phenons was indicated while, at the same time, the shared characteristics warranted their inclusion in the genus *Acinetobacter*.

Phenon 1

At the 76.5 % level of similarity, all but two of the strains in phenon 1 could be divided into three smaller clusters, termed 'subphenons' a, b and c (Fig. 1). Subphenon 1a members were distinguished by the production of a diffusible brown pigment. Most isolates in subphenon 1c, unlike other phenon 1 isolates, were able to tolerate 0.1 % sodium lauryl sulphate, and could utilize only a narrow range of organic compounds as sole carbon source. Subphenon 1b was characterized by a marked nutritional versatility.

Among the 87 members of phenon 1 were four reference strains, 82 aquatic isolates and the one hen manure isolate. The reference strains were *Achromobacter venenosum* NCIB9022,

Table 5. Comparison of base ratios among phenons and ungrouped strains

Strain allocation and designation	Strain no.*	Reference	% G+C
Phenon 1			
<i>Aci. calcoaceticus</i>	ATCC17925	Johnson, Anderson & Ordal (1970)	46
<i>Acinetobacter</i> sp.	PS98		45.8
Phenon 2			
<i>Aci. calcoaceticus</i>	ATCC17903	Johnson <i>et al.</i> (1970)	41
<i>Aci. calcoaceticus</i>	ATCC15308	De Ley (1968)	42.1
<i>Achromobacter</i> sp.	NCIB8250	Johnson <i>et al.</i> (1970)	42.2
<i>Acinetobacter</i> sp.	PS140		43.4
<i>Acinetobacter</i> sp.	PS97		43.6
<i>Acinetobacter</i> sp.	PS450		44.6
<i>Aci. calcoaceticus</i>	ATCC17924	Johnson <i>et al.</i> (1970)	45
<i>Aci. lwoffii</i>	ATCC15309	De Ley (1968)	45.6
Ungrouped strains			
<i>Mor. bovis</i>	NCTC8561	Bövre (1968)	42.5
<i>Alc. faecalis</i>	NCIB8156	Colwell & Mandel (1964)	54.8
<i>Ps. alcaligenes</i>	NCTC8769	Leifson & Mandel (1966)	66.0

* PS98, PS140, PS97 and PS450 refer to aquatic isolates from the present study.

Alcaligenes viscolactis NCIB8596, *Achromobacter lwoffii* NCIB9020 and *Acinetobacter calcoaceticus* ATCC17925.

Phenon 2

This second major group contained 204 strains all linked at similarity levels of 71.4 % or above. When clusters forming at the 75.5 % level were demarcated, a substructure similar to that in phenon 1 was observed (Fig. 1). Subphenons 2a and 2b showed internal similarities of 75.6 % or greater while 2c was more cohesive, clustering at a similarity level of 79.0 %. One strain could not be included in any subphenon since it joined subphenon 2a at a level of 72.2 %.

A few features differentiated these smaller clusters in phenon 2. Subphenons 2b and 2c, being much smaller than 2a, showed coccobacillary cellular morphology while, as a group, phenon 2 exhibited predominantly coccoid morphology. The general lack of acid production from arabinose and lactose, and positive lecithinase production also distinguished subphenon 2a. Greater nutritional versatility and a narrower pH range for growth (5.6 to 11.0) marked 2b. The nine isolates in subphenon 2c had narrower nutritional spectra than most other phenon 2 members.

The 204 isolates in the second phenon comprised 170 aquatic strains, 21 clinical isolates and 13 reference strains. Fig. 1 shows the reference and clinical strains according to subphenon.

Ungrouped strains

The 27 isolates excluded from phenons 1 and 2 comprised a heterogeneous selection of organisms representing a variety of genera. None of these strains fulfilled the diagnostic criteria previously listed for the genus *Acinetobacter*.

Among the 27 strains were 18 aquatic isolates, four reference strains and five clinical isolates. Most of the aquatic bacteria were oxidase-positive and probably belonged to the genus *Moraxella*. Two of the clinical isolates, *Mima polymorpha* var. *oxidans* and *Neisseria catarrhalis*, as well as the four reference strains, were also oxidase-positive. Base ratios published for the *Alc. faecalis* and *Ps. alcaligenes* strains (Table 5) supported their exclusion based on phenotypic characteristics.

DISCUSSION

Numerical analysis of the 318 strains characterized has revealed two major phenetic groups within the genus *Acinetobacter*. Although there has been little general accord on the taxonomy of this genus, the currently accepted classification groups all *Acinetobacter* strains into one species, *calcoaceticus*. To our knowledge, there has been no comprehensive numerical characterization of acinetobacters isolated from the environment; consequently it may well be that the existence of a valid second species, analogous to phenon 1, has been overlooked.

The concept of two groups within this genus supports the observations of Baumann *et al.* (1968) who found two clusters, denoted A and B, forming at the 61 % similarity level and seven subclusters at the 70 % level. Their group A appears to resemble phenon 2 in the present study, having several diagnostic characteristics and two reference strains in common (*Aci. calcoaceticus* ATCC17903 and 'Vibrio 01' NCIB8250). This cluster was proposed and accepted as the type species *Aci. calcoaceticus*. Phenon 1, being biochemically and nutritionally less active, corresponds closely to group B of Baumann *et al.* (1968). The species name *lwoffii* is associated with several reference strains in phenon 1 and has been suggested by Baumann *et al.* (1968) as an appropriate designation for their group B2. Henriksen (1973) deems it an acceptable epithet for this group of organisms should a separate species be proposed. Group B also tended to have higher G + C ratios than group A, again supported by preliminary data for the corresponding clusters in the present study (Table 5).

The subphenons delimited within both phenons probably represent biotypes within the genus *Acinetobacter* although it is interesting to note that 10 of the *Acinetobacter anitratum* strains were classified in phenon 2b and six of the *Mima polymorpha* isolates into phenon 2c, suggesting that these subphenons may denote previously recognized species.

The exclusion of the oxidase-positive organisms from both phenons, and the variety of genera classified into phenons 1 and 2, support transformation studies by Juni (1972). He found that auxotrophic mutants of *Aci. calcoaceticus* could be transformed by 265 oxidase-negative strains labelled *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Bacterium*, *Diplococcus*, *Herellea*, *Mima*, *Moraxella* and *Neisseria*. These names were concluded to be synonymous for a number of *Acinetobacter* biotypes with % G + C values ranging from 40.0 to 46.8. On the other hand, DNA from oxidase-positive *Neisseria*, *Moraxella*, *Pseudomonas* and *Alcaligenes* could not transform the recipient, indicating no relationship in this transformation system (Juni, 1972; Brooks & Sodeman, 1974).

Additional genetic studies are required to confirm the phenetic separation observed between phenons 1 and 2. The possible correlation between phenon and source of organisms should also be further investigated. Our findings indicate that this heterogeneous genus merits the addition of a second species to include the less reactive aquatic isolates. An appropriate species name may be *Acinetobacter lwoffii*.

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