Numerical Analysis of Phenotypic Features and Protein Gel Electropherograms of *Gluconobacter* Asai 1935 emend. mut. char. Asai, Iizuka, and Komagata 1964

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Ninety-eight *Gluconobacter* strains of various origins were examined by numerical analysis of 177 phenotypic features and by gel electrophoresis of soluble proteins. *Gluconobacter* was phenotypically quite different from *Acetobacter* and *Frateriella*. An extensive phenotypic description and a minimal description of the genus *Gluconobacter* are given. The genus *Gluconobacter* contained two groups, A and B, by both techniques. Phenons A and B could be differentiated only by the requirement for nicotinic acid and by their electrophoretic patterns. Protein electrophoresis showed clearly that *Gluconobacter* strains are genetically stable over several decades. The strains of all five subspecies of *Gluconobacter oxydans* cited in the Approved Lists of Bacterial Names (Skerman et al., Int. J. Syst. Bacteriol. 30:225–420, 1980) were distributed randomly over the phenotypic and electrophoretic clusters and subclusters, and the type strains of the subspecies all fell in phenon B. We conclude that the single species *Gluconobacter oxydans* should not be further divided into subspecies.

The acetic acid bacteria consist of the genera *Acetobacter* and *Gluconobacter*, which are both characterized by their capacity to oxidize ethanol to acetic acid. The polarly flagellated *Gluconobacter* does not oxidize lactate and acetate to CO₂ and water, whereas the peritrichously flagellated *Acetobacter* does (13, 21, 23, 39).

Recently, Gillis and De Ley (24) demonstrated a very high similarity between the ribosomal ribonucleic acid (rRNA) cistrons from *Acetobacter* and *Gluconobacter* and proposed that both genera be placed in the family *Acetobacteraceae*. This is supported by extensive phenotypic similarities between both genera. Consequently, *Gluconobacter* and *Acetobacter* will be classified together in the family *Acetobacteraceae* in the ninth edition of *Bergey's Manual of Determinative Bacteriology*.

The generic name *Gluconobacter* was introduced by Asai (3). Unfortunately, his paper, written in Japanese, remained unknown in the West. The classification and nomenclature of this genus have been discussed extensively by De Ley and Frateur (20). In *Bergey's Manual of Determinative Bacteriology* (21), the type and sole species *Gluconobacter oxydans* was subdivided into four subspecies: *G. oxydans*, *G. industrius*, *G. suboxydans*, and *G. melanogenes*. These subspecies seemed justified at that time for practical and historic reasons. The system described in *Bergey's Manual of Determinative Bacteriology* (21) constituted a considerable

simplification of a complicated older system of groups and species, and reduced the number of species to one. A fifth subspecies, *Gluconobacter oxydans* subsp. *sphaericus*, was added by Ameyama (1). The names of these five subspecies were preserved in the Approved Lists of Bacterial Names (48).

Loitianskaya et al. (40) applied numerical taxonomical methods to 136 phenotypic characters of 56 strains of acetic acid bacteria, including 15 *gluconobacters*. These authors found that the *Acetobacter* and *Gluconobacter* strains formed two well-separated groups, clustering at the simple matching coefficient (S_{SM}) of 75%. The results supported the existence of the species *Gluconobacter oxydans* within the genus *Gluconobacter* but cast doubt on the subspecies proposed in *Bergey's Manual of Determinative Bacteriology* (21).

Gillis and De Ley (24) found that authentic *Gluconobacter* and *Acetobacter* strains constituted well-defined and separate areas on the similarity map of the hybrids formed between their deoxyribonucleic acids (DNAs) and the 16S [¹⁴C]rRNA fraction of *Gluconobacter oxydans* subsp. *oxydans* NCIB 9013. On the basis of similar studies, a new genus *Frateriella* was proposed for strains previously identified as "*Acetobacter aurantius*" (52).

In this study, 177 phenotypic features were compared and used to cluster 98 *Gluconobacter*, 17 *Acetobacter*, and 7 *Frateriella aurantia* strains

by numerical analysis. The *Acetobacter* and *Frateruia* strains were included as controls only. We also compared and clustered *Gluconobacter* strains by polyacrylamide gel electrophoresis of their soluble proteins (32). It was our aim to give an extensive phenotypic description of the genus *Gluconobacter*, to determine the degree of its phenotypic and genetic homogeneity, to propose minimal standards for the genus, and to improve its classification. A similar study of a large collection of *Acetobacter* strains is in progress.

MATERIALS AND METHODS

Bacterial strains. The 98 *Gluconobacter*, 17 *Acetobacter* and 7 *Frateruia* strains used are listed in Table 1. They were grown and maintained either on GYC medium containing 5% D-glucose, 1% yeast extract (Oxoid Ltd.), 3% CaCO₃, and 2.5% agar or on MYP medium containing 2.5% D-mannitol, 0.5% yeast extract (Oxoid), 0.3% peptone (Oxoid), and 2.5% agar. The temperature for growth was always 28°C. The standard medium (SM) consisted of 0.5% yeast extract and 5% D-glucose.

Nutritional, physiological, and biochemical tests. A total of 177 phenotypic features were tested. Most tests have been described previously (52). Resistance towards antibiotics was tested by the Kirby-Bauer method (9), using Oxoid antibiotic disks. The effect of different concentrations of metals and dyes on the growth of *Gluconobacter* was tested in a basal medium containing 0.05% yeast extract (Oxoid), 0.3% vitamin-free Casamino Acids (Difco Laboratories), 0.1% D-glucose, 0.1% D-mannitol, 0.1% calcium-DL-lactate, and 2.5% agar (Oxoid no. 1). The medium of Asai et al. (5) was used to detect acid formation from different carbon sources. After 7 days of incubation at 28°C, the pH was measured. The utilization of different carbon sources for growth was tested on a solid medium containing 0.5% yeast extract (Oxoid), 0.3% vitamin-free Casamino Acids (Difco), 2.5% agar (Oxoid no. 1), and 0.3% of the carbon source. Growth factor requirements, the utilization of single L-amino acids as the sole sources of nitrogen, and the utilization of L-alanine, L-aspartic acid, L-glutamic acid, or L-proline as the sole sources of both carbon and nitrogen were tested as described before (26, 27). The formation of acetyl methyl carbinol in a medium containing 0.1% yeast extract and 1% calcium-DL-lactate was detected with Barritt's reagent (8) after 7 days of incubation.

Numerical analysis. Nearly all of the 177 phenotypic characters existed in one of two mutually exclusive states and were coded as 2 (positive) or 1 (negative). Multistate characters, such as pigmentation or colony morphology, were divided into several mutually exclusive states (49). For some strains, a few tests were missing (an average of 0.5 tests per strain), and such noncomparable results were coded as 0 (zero). The final $n \times t$ matrix comprised data for 122 strains and 188 unit characters. The S_{SM} and the Jaccard coefficient were calculated with the computer program of Bonham-Carter (11), adapted for the Siemens 7541 (BS 2000) computer of the Centraal Digitaal Rekencentrum, Rijksuniversiteit, Gent, Belgium. The strains were clustered according to the unweighted average

pair-group method (49), using the Clustan 1C program (64).

Polyacrylamide gel electrophoresis of soluble proteins and numerical analysis of protein electropherograms. Cells were grown in Roux bottles on GYC medium for 40 h at 28°C. Cell extracts were prepared and dialyzed as described previously (52). Polyacrylamide gel electrophoresis, densitometry and photography of the stained gels, normalization of the densitometric tracings, calculation of the Pearson product moment correlation coefficient r , and unweighted average linkage clustering were performed as described previously (31). At least 30% of the strains were grown in duplicate. Some strains, existing as duplicated cultures in different culture collections, were examined separately. For each protein extract at least three separate electrophoretic runs were performed.

RESULTS

Numerical analysis of morphological, biochemical, and physiological features. A dendrogram representing the results of the numerical analysis of the phenotypic features with the S_{SM} is given in Fig. 1. The seven *Frateruia aurantia* strains (52) grouped together above 76% S_{SM} and separated clearly from the *Acetobacter* and *Gluconobacter* strains. *Gluconobacter* separated from *Acetobacter* at 68% S_{SM} . The 98 *Gluconobacter* strains investigated were divided into two phenons, A (50 strains) and B (47 strains). One *Gluconobacter oxydans* subsp. *suboxydans* strain, i.e., IAM 1832 t₁, could not be assigned to either one of these phenons. No clearly separated subphenons could be distinguished within phenons A and B. The position of each *Gluconobacter* strain within the dendrogram is indicated in Table 1 and Fig. 1.

The same phenons A and B were retrieved after analyzing the data with the Jaccard coefficient (figure not given). Some strains shifted their position somewhat, but there were no fundamental differences between Jaccard coefficient and S_{SM} dendrograms.

For the different groups of phenotypic tests, the following reproducibilities were obtained: growth on carbon sources, 95%; acid from carbon sources, 94%; growth on single L-amino acids as the sole nitrogen source, 93%; growth factor requirements, 97%; growth in the presence of metals and dyes, 97%. Independent retesting of the whole set of 177 phenotypic features on strain ATCC 15180 showed a reproducibility of 93%.

All *Gluconobacter* strains displayed the following 20 features: occurrence of single cells; growth on 5% D-glucose–0.5% yeast extract at 28°C; final pH on D-xylose below 4.5; final pH on D-ribose below 5.9; resistance per disk to 10 µg of gentamicin, 10 U of bacitracin, 10 U of penicillin G, 10 µg of erythromycin, 2 µg of lincomycin, 10 µg of colistin sulfate, 10 µg of

TABLE 1. Strains used: information on their source, isolation, and position in our phenotypic and electrophoretic dendrograms

Name	Strain no. ^a	Position in dendrogram ^b		Source	Place and year of isolation	Reference or isolated by:
		Pheno- typic	Electro- phoretic			
<i>Gluconobacter oxydans</i> subsp. <i>oxydans</i>	IFO 3287	B	B'2	Flower of <i>Liatris scariosa</i>	Kyoto, Japan, 1941	K. Kondô, Shizuoka Women's College, Shizuoka, Japan
	ATCC 15178	A	A'1			Kyowa Fermentation Industrial Co. Ltd.
	ATCC 15179	A	A'4			Kyowa Fermentation Industrial Co. Ltd.
	ATCC 15180	A	A'4			Kyowa Fermentation Industrial Co. Ltd.
<i>G. oxydans</i> subsp. <i>suboxydans</i>	NCIB 9013 ^{Tc}	B	B'1	Beer		
	NCIB 9014	B	B'2	Cider		
	IFO 3251 t ₁ and t ₂	A	A'4	Flower of <i>Dahlia</i>	Kyoto, Japan, 1941	K. Kondô
	IFO 3253	A	A'4	Flower of <i>Dahlia</i>	Kyoto, Japan, 1941	K. Kondô
	IFO 3255	A	A'4	Strawberry (<i>Fragaria chilonensis</i> var. <i>ananassa</i>)	Osaka, Japan, 1941	K. Kondô
	IFO 3257	A	A'4	Fruit of <i>Prunus tomentosa</i>	Osaka, Japan, 1941	K. Kondô
	IFO 3258 t ₁ and t ₂	A	A'4	Fruit of <i>Prunus tomentosa</i>	Osaka, Japan, 1941	K. Kondô
	IFO 3262 t ₁ and t ₂	A	A'4	Strawberry	Osaka, Japan, 1941	K. Kondô
	IFO 3263	A	A'4	Strawberry	Osaka, Japan, 1941	K. Kondô
	IFO 3264	A	A'4	Strawberry	Osaka, Japan, 1941	K. Kondô
	IFO 3265	A	A'1	Strawberry	Osaka, Japan, 1941	K. Kondô
	IFO 3266 t ₁	B	B'2	Strawberry	Osaka, Japan, 1941	K. Kondô
	IFO 3267 t ₁ and t ₂	A	A'1	Cherry	Osaka, Japan, 1941	K. Kondô
	IFO 3268 t ₁ and t ₂	A	A'4	Cherry	Osaka, Japan, 1941	K. Kondô
	IFO 3269 t ₁ and t ₂	A	A'3	Cherry	Osaka, Japan, 1941	K. Kondô
	IFO 3270	A	A'1	Cherry	Osaka, Japan, 1941	K. Kondô
	IFO 3271	A	A'3	Fruit of <i>Myrica rubra</i>	Nishinomiya, Japan, 1941	K. Kondô
	IFO 3273	B	B'2	Fruit of <i>Myrica rubra</i>	Nishinomiya, Japan, 1941	K. Kondô
	IFO 3274	A	A'1	Fruit of <i>Myrica rubra</i>	Nishinomiya, Japan, 1941	K. Kondô
	IFO 3276	A	A'2	Flower of <i>Rheum undulatum japonica</i>	Kyoto, Japan, 1941	K. Kondô
	IFO 3285 t ₁ and t ₂	A	A'1	Fruit of <i>Eriobotrya japonica</i>	Osaka, Japan, 1941	K. Kondô
	IFO 3286	A	A'4	Fruit of <i>Eriobotrya japonica</i>	Osaka, Japan, 1941	K. Kondô

TABLE 1—Continued

Name	Strain no. ^a	Position in dendrogram ^b		Source	Place and year of isolation	Reference or isolated by:
		Pheno- typic	Electro- phoretic			
<i>G. oxydans</i> subsp. <i>industrius</i>	IFO 3289	A	A'4	Persimmon	Osaka, Japan, 1941	K. Kondô
	IFO 3290	A	A'4	Persimmon	Osaka, Japan, 1941	K. Kondô
	IFO 3291	A	A'4	Persimmon	Osaka, Japan, 1941	K. Kondô
	IFO 12528 ^{1c}	B	B'2	Beer (Amstel)		36
	NCIB 621 ^{1c}	B	B'2	Beer (Amstel)		36
	NCIB 3734	B	B'1	Beer		F. de Leeuw, LMD
	NCIB 6723	B	B'2			
	NCIB 7069	B	B'2	Beer (Amstel)		36
	NCIB 8035	B	B'2	Beer (Amstel)		36
	NCIB 9108	A	A'2	Beer	The Netherlands	50
	NCIB 9119	B	B'2	Beer (Amstel)		F. de Leeuw
	NCIB 9137	B	B'1	Probably persimmon	Tokyo, Japan, 1934	T. Asai, Japan
	NCIB 9498	B	B'2	Beer (Amstel)		36
	LMD 29.7 t ₁ and t ₂	B	B'1	Beer	Leuven, Belgium, 1929	23
	LMD 53.12	B	B'2	Sour porridge	South Africa, 1950	
	IAM 1815	A	A'4			
	IAM 1832 t ₁		A'1	Apple	Japan, 1935	3
	IAM 1839	B	A'1			
	IAM 1841	A	A'3			
	IAM 1842	B	A'1			
	CCM 1792	A	A'1	Strawberry (<i>Fragaria chilonensis</i> var. <i>ananassa</i>)	Osaka, Japan, 1941	K. Kondô
	CCM 2365	B	B'2	<i>Dahlia coccinea</i>		37
	C 115 A	B	B'2	Beer	Ireland	R. B. Gilliland, Arthur Guinness Research Lab., Dublin
	CN 1221	B				Obtained from Wellcome Research Laboratories
	IFO 3260	A	A'3	Fruit of <i>Myrica rubra</i>	Nishinomiya, Japan, 1941	K. Kondô
	NCIB 4943	B	B'1	Beer	United Kingdom	47
	NCIB 5595	B	B'2	Beer		
	NCIB 8131	B	B'1	Top-fermentation beer	Leuven, Belgium, 1929	62
	NCIB 8624	A	A'3	Baker's yeast	Delft, The Netherlands, 1924	23
	LMD 24.1 ^{1c}	B	B'1	Beer		R. Nagel, LMD
	LMD 32.1	B	B'1	Beer	Delft, The Netherlands, 1932	M. A. van Overeem, LMD

<i>G. oxydans</i> subsp. <i>melanogenes</i>	LMD 37.1 t ₁ and t ₂	A	A'3	Beer	Delft, The Netherlands, 1937	J. Bosman, Botanisch Laboratories, Leiden, The Netherlands
	LMD 53.11 t ₁ and t ₂	A	A'2	Beer	Leiden, The Netherlands	
	CCM 2369	B	B'1			
	NCIB 8084	B	B'2			
	NCIB 8085	B	B'2			
	NCIB 8086	B	B'2			
	IFO 3243	B	B'2			
	IFO 3244	B	B'2			
	IFO 3293	B	B'	Dried persimmon	Osaka, Japan, 1941	
	IFO 3294	A	A'1	Dried persimmon	Osaka, Japan, 1941	
<i>G. oxydans</i> subsp. <i>sphaericus</i>	LMD 29.2 ^{Tc} 49	B	B'2	Beer	1928	K. Kondô K. Kondô
						Obtained from K. Kimmitt, H. P. Sauce Ltd., Birmingham, United Kingdom
	116	B	B'			Obtained from A. Stouthamer, University of Amsterdam
	IFO 12467 ^{Tc}	B	B'	Grapes	Japan, 1966	1
	IFO 3246 ^d	B	B'2	<i>Lilium auratum</i>	Japan	37
	NCIB 6426 ^d	B	B'1	Ropy sample of top- fermented beer	United Kingdom, 1943	62
	NRRL B-1810 ^d	B	B'	Fermenting Agave juice	Lake Lerma, Mexico, 1950	25
	LMG 76.1	A	A'1	Rotting pear	Gent, Belgium, 1976	J. Swings, LMG
	LMG 76.5	A	A'4	Rotting pear	Gent, Belgium, 1976	J. Swings
	LMG 76.7	A	A'4	Rotting pear	Gent, Belgium, 1976	J. Swings
<i>G. oxydans</i>	LMG 76.8	B	B'	Rotting pear	Gent, Belgium, 1976	J. Swings
	LMG 76.9	B	B'	Rotting apple	Gent, Belgium, 1976	J. Swings
	LMG 79.1	A	A'4	Rotting apple	Gent, Belgium, 1976	J. Swings
	LMG 79.37	B	B'	Bee		38
	LMG 79.87	A	A'2	Bee		38
	7-490	A	A'1	Orange lemonade	The Netherlands	G. Kolfshoten, Naarden, The Netherlands
	7-501	B	B'1	Cider lemonade	The Netherlands	G. Kolfshoten
	7-582	B	B'1	Lemon lemonade	The Netherlands	G. Kolfshoten
	7-712	A	A'1	Raspberry stilldrink	The Netherlands	G. Kolfshoten
	7-827	A	A'1	Cassis lemonade	The Netherlands	G. Kolfshoten
	7-903	B	B'	Orange lemonade	The Netherlands	G. Kolfshoten

TABLE 1—Continued

Name	Strain no. ^a	Position in dendrogram ^b		Source	Place and year of isolation	Reference or isolated by:
		Pheno- typic	Electro- phoretic			
<i>Acetobacter aceti</i> subsp. <i>liquefaciens</i>	IFO 12388 ^{Tc} NCIB 9417			Dried fruit of <i>Diospyros kaki</i> (persimmon)	Japan, 1935 Japan	3
<i>A. aceti</i> subsp. <i>xylinum</i>	NCIB 8246 NCIB 11664			Vinegar	Jerusalem, Israel, 1936 Paris, 1896	7 10
<i>A. aceti</i> subsp. <i>orleanensis</i>	NCIB 8622 ^{Tc} NCIB 8746			Beer Vinegar	Belgium, 1927 Jerusalem, Israel, 1936	23 7
<i>A. aceti</i> subsp. <i>aceti</i>	NCIB 8621 t ₁ ^{Tc} LMG 5			Beechwood shavings in vinegar generator Beer	Belgium, 1927	M. Arends 23
<i>A. pasteurianus</i> subsp. <i>lovaniensis</i>	NCIB 8620 ^{Tc}			Sewage on soil	Beckvevoort, Belgium, 1929	23
<i>A. pasteurianus</i> subsp. <i>estunensis</i>	NCIB 8935 ^{Tc} LMG E			Cider Cider	Bristol, United Kingdom Bristol, United Kingdom	12 12
<i>A. pasteurianus</i> subsp. <i>pasteurianus</i>	NCIB 7113 LMD 22.1 t ₁ ^{Tc}			Beer		
<i>Acetobacter</i> sp.	LMG 76.10 NCPFB 461 ^d NCPFB 462 ^d NCPFB 463 ^d			Rotting apple Rotting apple Rotting apple Rotting apple	Gent, Belgium, 1976 United Kingdom, 1955 United Kingdom, 1955 United Kingdom, 1955	J. Swings 14 14 14
<i>Frateruria aurantia</i> ^c	IFO 3245 ^{Tc} IFO 3247 IFO 3249 IFO 13328			<i>Lilium auratum</i> <i>Lilium auratum</i> <i>Lilium auratum</i> Fruit of <i>Rubus parvifolius</i> (raspberry)	Japan Japan Japan Yuata, Japan	37 37 37 65
	IFO 13330			Fruit of <i>Rubus parvifolius</i> (raspberry)	Yuata, Japan	65
	IFO 13331			Fruit of <i>Rubus parvifolius</i> (raspberry)	Yuata, Japan	65

IFO 13333	Fruit of <i>Rubus parvifolius</i> (raspberry)	Yuata, Japan	65
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^a Abbreviations used: ATCC: American Type Culture Collection; CCM: Czechoslovak Collection of Microorganisms; IAM: Institute for Applied Microbiology; IFO: Institute for Fermentation, Osaka; LMD: Laboratorium voor Mikrobiologie, Technische Hogeschool, Delft; NCIB: National Collection of Industrial Bacteria; NRRL: Northern Regional Research Laboratory; NCPPB: National Collection of Plant Pathogenic Bacteria; LMG: our laboratory. Some strains showed two different stable colony types upon plating: these are t_1 and t_2 .

^b The symbols used are the phenons and (sub)clusters (Fig. 1 and 2).

^c Type strains according to the Approved Lists of Bacterial Names (48). Some of these type strains have synonymous strain numbers in other collections: NCIB 621^T = LMD 23.2^T = ATCC 621^T = IFO 12528^T; NCIB 9013^T = ATCC 19357^T; LMD 24.1^T = Frateur II0^T; NCIB 8622^T = ATCC 12876^T; NCIB 8620^T = ATCC 12875^T; NCIB 8935^T = ATCC 23753^T; IFO 12388^T = IAM 1834^T.

^d Some culture collection strains were misnamed: "*A. lermiae*" NRRL B-1810, "*A. acetii* subsp. *orleanensis*" NCIB 6426, and "*A. aurantium*" IFO 3246 belong to *Gluconobacter* (24). "*Pseudomonas melophthora*" NCPPB 461 and NCPPB 462, and "*P. pomi*" NCPPB 463 belong to *Acetobacter* (22, 24).

fucidin, 200 µg of nitrofurantoin, 10 µg of methicillin, 30 µg of neomycin, 300 U of polymyxin B, 100 µg of sulphafurazole; presence of catalase; formation of 2-ketogluconic acid from D-glucose; ketogenesis from glycerol; and growth factor requirements with D-mannitol as the carbon source.

All *Gluconobacter* strains lacked the following 39 features: growth on 0.5% yeast extract broth, 0.5% peptone broth, or nutrient broth; growth in anaerobic conditions; reaction in litmus milk; growth on Frateur's modified Hoyer medium with ethanol with or without growth factors; growth on 5% D-glucose–0.5% yeast extract at 4°C; growth on 5% D-glucose–0.5% yeast extract and either 2% NaCl or 10% ethanol; final pH on starch or lactose below 5.9; growth on ethanediol, galactitol, D-arabinose, L-rhamnose, D-mannose, L-lyxose, cellobiose, lactose, starch, sodium formate, sodium oxalate, sodium citrate, sodium malonate, sodium-potassium tartrate, calcium-DL-glycerate, or sodium-L-malate as the sole source of carbon; growth on L-alanine, L-aspartic acid, L-glutamic acid, or L-proline as the source of both carbon and nitrogen; presence of oxidase; reduction of nitrate; formation of indole; formation of H₂S; gelatin liquefaction; oxidation of lactate or ethanol to CO₂ and water.

All other features for which the *Gluconobacter* strains were studied gave differential results (Table 2).

Numerical comparison of gel electrophoretic protein patterns. Protein electropherograms were prepared for all of the *Gluconobacter* strains listed in Table 1, except strain CN 1221. More than 400 normalized electropherograms of 97 *Gluconobacter* strains were compared by numerical analysis. The reproducibility limits were above $r = 0.95$ for 82 strains and above $r = 0.93$ for the remaining 15 strains. The most typical electropherogram of each strain (53) was used for the final clustering presented as a simplified dendrogram in Fig. 2. The protein electrophoretic heterogeneity of the *Gluconobacter* strains is obvious; at $r = 0.75$ two electrophoretic clusters A' (53 strains) and B' (44 strains) could be delineated. With three exceptions, all strains belonging to the phenotypic phenon A belonged in the electrophoretic cluster A' and all strains from phenon B fell in cluster B'. Within clusters A' and B', respectively, four (A'1 to A'4) and two (B'1 and B'2) electrophoretic subclusters could be recognized at $r = 0.90$. The electropherograms of the strains belonging to each subcluster were always very similar upon visual inspection. Phenotypic counterparts of these electrophoretic subclusters could not be detected in phenons A and B. The position of each *Gluconobacter* strain within the electro-

phoretic clusters A' and B' and their subclusters can be found in Table 1. The following 12 strains of electrophoretic cluster B' could not be assigned to any of the subclusters: *G. oxydans* subsp. *suboxydans* NCIB 9137 and LMD 29.7 t₁ and t₂, *G. oxydans* subsp. *melanogenes* strains 49, 116, and IFO 3293, *G. oxydans* subsp. *sphaericus* IFO 12467^T, *G. oxydans* NRRL B-1810, and *G. oxydans* strains 7-903 and LMG 76.8, 76.9, and 79.37. Normalized electropherograms of 30 representative *Gluconobacter* strains are shown in Fig. 3.

DISCUSSION

The named strains of the genus *Gluconobacter* will be considered first. It is obvious that

they are quite different from *Frateruia* and from *Acetobacter* (Fig. 1).

Morphological, biochemical, and physiological features of *Gluconobacter*. Features which occur or are lacking in all *Gluconobacter* strains have been reported above.

The majority of the strains (76%) were found to be nonmotile in our test conditions. The application of classical staining procedures (39, 42) revealed that all motile strains examined bore polar flagella. The difficulty of staining the flagella of acetic acid bacteria for light microscopy has been reported by several authors, e.g., Passmore (Ph.D. thesis, University of Bristol, Bristol, England, 1973). According to Loitian-skaya et al. (41), the type of flagellation is not a

FIG. 1. Dendrogram obtained by numerical analysis of 177 phenotypic features of 98 *Gluconobacter*, 17 *Acetobacter*, and 7 *Frateruia* strains with the S_{SM} and the unweighted average pair-group method. The numbers of strains are given in parentheses.

TABLE 2. Phenotypic features which are not uniformly positive or negative in 98 *Gluconobacter* strains

Feature	Percentage of positive <i>Gluconobacter</i> strains			
	Phenon A (50 strains)	Phenon B (47 strains)	IAM 1832 t ₁ (1 strain) ^a	Total (98 strains)
Morphology after 10 days on GYC medium ^b				
Moderate to abundant growth	100	77	1	89
Luxuriant colony	2	28	0	14
Pale colony colour	84	55	1	70
Pink colony colour	32	19	0	26
Soluble brown pigment	0	21	0	10
Colony diameter <3 mm	54	85	1	69
Flat colony	26	45	0	35
Regularly edged colony	54	77	1	66
Cell morphology of 2- to 3-day-old cultures				
Gram-negative cells	90	89	1	90
Gram-variable cells	10	11	0	10
Rods	80	77	0	78
Ovoids	34	49	1	42
Tapered cells	30	4	0	17
Filaments	64	45	0	54
Chains	6	21	0	13
Cells in pairs	94	96	1	95
Enlarged, irregular involution forms	46	34	0	40
Curved cells	48	38	0	43
Cell diameter $\geq 0.7 \mu\text{m}$	22	26	0	23
Motile cells	16	34	0	24
Acid production from: ^c				
Ethanol	86	89	1	88
<i>n</i> -Propanol	94	96	0	94
<i>n</i> -Butanol	83	96	NT	88
<i>n</i> -Amyl alcohol	57	56	0	56
Glycerol	96	79	0	87
<i>meso</i> -Erythritol	98	94	1	96
D-Mannitol	92	85	0	87
D-Glucitol	68	70	0	68
<i>meso</i> -Inositol	28	4	1	17
D-Arabinose	98	100	1	99
L-Rhamnose	4	2	0	3
D-Galactose	84	94	1	89
D-Glucose	100	98	1	99
D-Fructose	92	94	1	93
D-Mannose	94	96	1	95
L-Sorbose	40	54	0	46
Cellobiose	6	2	0	4
Maltose	78	98	1	88
Sucrose	76	53	0	64
Raffinose	6	4	0	5
Dextrin	4	0	0	2
Growth on:				
Frater's Hoyer medium				
With glucose and growth factors	88	62	0	74
With D-mannitol and growth factors	100	89	1	95
SM ^d containing				
0.5% NaCl	70	66	0	67
1% NaCl	40	26	0	33
1% ethanol	88	94	0	90
2% ethanol	88	89	0	88
5% ethanol	20	64	0	41
SM ^d at 34°C	40	43	0	41

TABLE 2—Continued

Feature	Percentage of positive <i>Gluconobacter</i> strains			
	Phenon A (50 strains)	Phenon B (47 strains)	IAM 1832 t ₁ (1 strain) ^a	Total (98 strains)
37°C	2	0	0	1
pH 3.6	86	89	1	88
0.5% yeast extract containing				
20% D-glucose	62	38	0	50
25% D-glucose	36	17	0	27
30% D-glucose	18	2	0	10
Carbon sources for growth				
Methanol	2	0	0	1
Ethanol	10	11	1	11
<i>n</i> -Propanol	10	9	0	9
Glycerol	98	83	1	91
<i>meso</i> -Erythritol	58	74	0	65
<i>meso</i> -Ribitol	82	11	0	47
<i>meso</i> -Xylitol	96	15	0	47
L-Arabitol	78	4	0	42
<i>meso</i> -Inositol	40	9	0	24
D-Mannitol	98	89	1	94
D-Glucitol	98	89	1	94
L-Arabinose	2	2	0	2
D-Ribose	20	2	0	11
D-Xylose	2	2	0	2
D-Fructose	92	83	0	87
D-Galactose	0	2	0	1
D-Glucose	96	62	0	79
Calcium-D-gluconate	52	36	1	45
L-Sorbose	56	36	0	46
D-Lyxose	2	0	0	1
D-Fucose	18	13	0	15
Maltose	18	30	0	23
Sucrose	34	13	0	23
Raffinose	8	0	0	4
Dextrin	0	7	0	3
Sodium acetate	0	0	1	1
Sodium-DL-lactate	6	7	0	6
Biochemical reactions				
Production of 5-ketogluconic acid from D-glucose	96	94	1	95
Production of 2,5-diketogluconic acid from D-glucose	0	35	0	16
Ferric chloride reaction on				
D-Glucose	0	32	0	16
D-Fructose	89	87	1	89
D-Galactose	2	0	0	1
Ketogenesis from:				
D-Mannitol	98	98	1	98
D-Glucitol	100	98	1	99
Production of acetylmethylcarbinol from calcium-DL-lactate	78	83	1	81
Single L-amino acid as sole source of nitrogen				
L-Alanine	92	45	1	69
L-Arginine	94	32	1	64
L-Asparagine	100	89	1	95
L-Aspartic acid	98	68	1	84
L-Cysteine	66	15	1	42

TABLE 2—Continued

Feature	Percentage of positive <i>Gluconobacter</i> strains			
	Phenon A (50 strains)	Phenon B (47 strains)	IAM 1832 t ₁ (1 strain) ^a	Total (98 strains)
L-Glutamine	98	89	1	94
L-Glutamic acid	98	77	1	88
L-Glycine	40	23	0	32
L-Histidine	46	60	0	52
L-Isoleucine	92	38	1	66
L-Leucine	80	2	0	42
L-Lysine	86	32	1	60
L-Methionine	78	34	1	56
L-Phenylalanine	88	26	1	58
L-Proline	96	62	1	80
L-Serine	48	36	1	43
L-Threonine	56	28	0	42
L-Tryptophan	78	36	1	58
L-Tyrosine	72	23	0	48
L-Valine	38	0	0	19
Growth factor requirements in the presence of D-mannitol as carbon source				
p-Aminobenzoic acid	2	19	1	11
Thiamine	0	26	1	13
Nicotinic acid	0	98	1	48
Pantothenic acid	100	89	1	95
Other growth factors	0	6	1	4
Resistance to antibiotics				
Kanamycin (30 µg)	48	19	0	34
Streptomycin (10 µg)	62	23	0	43
Tetracycline (30 µg)	6	7	0	6
Novobiocin (30 µg)	4	7	1	6
Ampicillin (10 µg)	86	77	1	82
Nalidixic acid (30 µg)	94	87	1	91
Chloramphenicol (30 µg)	98	96	0	96
Cephaloridin (25 µg)	84	96	1	90
Growth in the presence of metals and dyes				
0.001% Malachite green	98	49	0	74
0.0001% Crystal violet	82	51	1	67
0.001% Brilliant green	94	46	0	70
0.01% Cd(CH ₃ · COO) ₂ · 2H ₂ O	28	32	0	30
0.01% CoSO ₄ · 7H ₂ O	54	13	1	35
0.001% HgCl ₂	42	26	0	34
0.001% Ti-OOC · CH ₃	92	79	0	85

^a Since only one strain was available, the results are represented by 0 (feature absent) or 1 (feature present). NT, not tested.

^b GYC medium. See text.

^c A final pH below 5.9 was considered as positive for acid formation for all carbon sources, except for D-glucose, D-xylose, and D-galactose for which pH 4.5 was taken.

^d SM medium. See text.

reliable character among the acetic acid bacteria and is of little use for the differentiation of *Acetobacter* and *Gluconobacter*.

Gram variability and the formation of enlarged, irregular cells ("hypertrophische Zellformen" [30]; "involution forms") are not restricted to older cultures as previously thought. These features occurred in 10% and 40%, respectively,

of the *Gluconobacter* strains. Passmore (Ph.D. thesis, University of Bristol, Bristol, England, 1973) has suggested that involution forms might be a stage in a life cycle.

The presence of a water-insoluble pink pigment has been used as a diagnostic feature in previous classifications (3, 55, 59–61). Frateur (23) noted that the formation of a pink pigment is

not a reliable taxonomic feature. It can also be confused with the presence of cytochromes. Consequently, De Ley and Frateur (21) did not use it as a diagnostic characteristic. Our results (Table 2) show that pink pigments were formed by strains from both phenons A and B. In all strains tested, the formation of a water-soluble, coffee-brown pigment correlated with the production of 2,5-diketogluconic acid and γ -pyrones from D-glucose.

Nearly all strains possessed ketogenic activity towards the sugars and polyalcohols tested. The formation of 2-keto- and 5-ketogluconic acid from D-glucose, found in 100 and 95%, respectively, of the strains, is a reliable generic feature of *Gluconobacter*.

More than 85% of the *Gluconobacter* strains formed acid from ethanol, 1-propanol, 1-butanol, glycerol, meso-erythritol, D-mannitol, D-xylose, D-arabinose, D-ribose, D-glucose, D-galactose, D-fructose, D-mannose, and maltose. Acid formation from L-rhamnose, cellobiose, lactose, raffinose, dextrin, and starch was only rarely observed, as less than 5% of the strains were positive. This confirms the data of Asai (3), Asai and Shoda (6), De Ley (18), Asai et al. (5),

and De Ley and Frateur (20, 21).

The formation of acetic acid from ethanol is the most striking common feature of acetic acid bacteria. In liquid media, however, only 88% of the *Gluconobacter* strains acidified ethanol. When Carr's procedure (13) on agar medium was used, only two of the 98 *Gluconobacter* strains, i.e., NCIB 9498 and IAM 1832 t_1 , failed to form acetic acid from ethanol. However, both strains formed some acid from ethanol in the liquid medium of Asai et al. (5). The former strain is a variant of strain NCIB 8036, which is itself a descendant of the type strain NCIB 621^T of *G. oxydans* subsp. *suboxydans*. Fig. 1 shows that NCIB 621^T and NCIB 9498 cluster together at 91% S_{SM} in the middle of phenon B. Gillis and De Ley (24) showed by DNA:rRNA hybridizations that strain NCIB 9498 belongs to the genus *Gluconobacter*. Its protein pattern is almost indistinguishable from the patterns of strain NCIB 621^T and NCIB 7069 (Fig. 3). We conclude that strain NCIB 9498 is a normal *Gluconobacter*, in spite of its poor ethanol oxidation. Strain IAM 1832 will be discussed below.

It appears that the ability to oxidize ethanol extensively to acetic acid is not necessarily an essential characteristic of *Gluconobacter*. For ethanol-oxidizing strains, a rapid, simple and accurate differentiation of *Gluconobacter* from *Acetobacter* is possible, as the former does not oxidize ethanol via acetate to CO₂ and water whereas the latter does.

Acid production from a carbohydrate does not imply that growth is sustained by it, e.g., whereas all strains acidified D-xylose (pH $\leq$ 4.50), only 2% were able to grow on it. As stated by Henneberg (30), "wenn auch Säuerung zu konstatieren war, konnte in verschiedenen Fälle keine Hautbildung wahrgenommen werden." The best carbon sources for growth of *Gluconobacter* were, in descending order: D-mannitol, D-glucitol, glycerol, D-fructose, and D-glucose.

As *Gluconobacter* strains have no complete tricarboxylic acid cycle enzyme sequence (15, 28, 29, 34, 35, 63), it is not surprising that no growth was obtained on the tricarboxylic acid cycle intermediates citrate and malate.

The majority of *Gluconobacter* strains were able to utilize L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, and L-proline as sole sources of nitrogen for growth. It is known that these amino acids play a key role in the nutrition of *Gluconobacter*. The majority of *Gluconobacter* strains (81%) did not grow with L-valine as the sole source of nitrogen. This phenomenon might be due to the inhibitory effect of this amino acid (17, 33; R. J. Shamberger, M.S. Thesis, Oregon State University, Corvallis, 1960). A discussion of the nitrogen requirements of *Gluconobacter*, *Acetobacter*, and *Frateruia*

FIG. 2. Simplified dendrogram of correlation coefficients r , grouped by the unweighted average pair-group method, showing the relationships between protein electropherograms of 97 *Gluconobacter* strains. Electrophoretic (sub)clusters are labeled as in the text and in Fig. 3. The number of strains is indicated in parentheses to the left of the subcluster label. Strain numbers are given for single strains.

has been given elsewhere (27).

The acid-tolerant character of *Gluconobacter* was stressed by Asai (3). We found that 88% of the strains grew at pH 3.6.

It has been reported that the members of the genus *Gluconobacter* tolerate 1 to 7% ethanol (3, 54; S. M. Passmore, Ph.D. thesis). These observations correspond well with our findings. In the presence of 1, 2, and 5% ethanol, 90, 88, and 41%, respectively, of the strains grew. Phenon B contained the most ethanol-resistant strains. This is not surprising as 20 strains from phenon B were isolated from alcohol-containing environments, e.g., beer, cider, and fermenting *Agave* juice.

Another striking feature of the genus *Gluconobacter* is its glucose tolerance, as 27% of the strains grew in the presence of 25% glucose. This confirms earlier observations (23, 56).

Heterogeneity within the genus *Gluconobacter*. With the exception of strains IAM 1842, IAM 1839, and IAM 1832 t_1 , every strain which clustered in either phenon A or B in the numerical taxonomic study also fell into the electrophoretic cluster A' or B', respectively (see Table 1). However, strains IAM 1842 and IAM 1839 belonged to the electrophoretic subcluster A'1, but

were members of phenon B.

This subdivision of *Gluconobacter* into two phenons A and B occurs on the basis of two different sets of criteria. They can be differentiated by two features only: differences in the electrophoretic protein profiles and in the requirement for nicotinic acid for growth. Nicotinic acid is required by 98% of the strains from phenon B and none of the strains from phenon A. This constitutes the sole phenotypic feature differentiating phenons A and B. A discussion of the growth factor requirements of *Gluconobacter* can be found elsewhere (26). Although the differentiation is never complete, there are other phenotypic features which tend to separate the phenons. These are the following.

(i) On the whole, strains of phenon A were better equipped to use the pentitols *meso*-ribitol, *meso*-xylitol, and L-arabitol as carbon sources.

(ii) The utilization of single L-amino acids as the sole source of nitrogen was more widespread among strains from phenon A than from phenon B. This is particularly true for L-leucine, which supported growth of 80% of the strains from phenon A, but only 2% from phenon B.

(iii) A water-soluble, coffee-brown pigment was formed by 21% of the strains from phenon

FIG. 3. Normalized protein electropherograms of 30 representative *Gluconobacter oxydans* strains. A'1, A'2, etc. refer to (sub)clusters of Fig. 2 and Table 1. Strain numbers refer to strains from Table 1.

B, but by no strain from phenon A. In *Bergey's Manual of Determinative Bacteriology* (21), this feature was reported for strains of the *G. oxydans* subspecies *industrius* and *melanogenes*.

(iv) Glucose tolerance was more obvious in phenon A than in phenon B. It should be added that phenon A contained 41 strains isolated from sugar-containing sources such as fruits, flowers, and soft drinks. The nature of the original environment seems to be reflected in the sugar and ethanol tolerance of the isolates.

(v) Resistance towards antibiotics, metals, and dyes was more widespread in phenon A.

Strain *G. oxydans* subsp. *suboxydans* IAM 1832 t_1 is a separate case; it is phenotypically quite different from phenons A or B but belongs electrophoretically to the subcluster A'1. Asai (4) discussed and identified this strain as a *Gluconobacter*. Our results confirm this, as it shares a large number of features with the other *Gluconobacter* strains. The single strain IAM 1832 t_1 can be partially differentiated by a number of features from both phenon A and phenon B (Table 2). Strain IAM 1832 t_1 is outstanding in that it produces little or no (according to the conditions) acetic acid from ethanol. One explanation for the lack of other strains resembling strain IAM 1832 might be that a number of *Gluconobacter* strains which oxidize ethanol poorly have been overlooked systematically by the common detection methods which select strains producing acid from ethanol.

As far as the internal structure of the phenons is concerned, the similarity matrix (not given), the dendrogram (Fig. 1), and Table 2 indicate that each phenon is rather heterogeneous. A clear-cut phenotypic separation of subphenons within A and B is impossible. The heterogeneity of the genus *Gluconobacter* is also obvious from the electropherogram (Fig. 2 and 3). Phenotypic differentiation between the electrophoretic subclusters was not possible.

Another indication of the heterogeneity within *Gluconobacter* is found in the relatively large mole percent guanine plus cytosine (mol% G+C) range in its DNA from 56 to 64% (24). It has been stated that the percent guanine plus cytosine span of most bacterial genera does not exceed 10% (19).

From phenons A and B, three and seven strains, respectively, were used in DNA:DNA hybridizations (M. Gillis, unpublished data). The following mean DNA relatednesses were found: 44.5% D (degree of DNA relatedness) for hybridizations of A strains with other A strains (2 hybridizations), 81% D for hybridizations of B strains with other B strains (14 hybridizations), and 30% D for hybridizations of A strains with B strains (13 hybridizations). Within these three series of hybridizations, the lowest DNA rela-

tednesses obtained were, respectively, 27% D, 40% D, and 16% D, confirming the genetic heterogeneity within *Gluconobacter oxydans*.

Taxonomy of *Gluconobacter*. *Gluconobacter* constitutes a well-defined genus that can be distinguished easily from *Acetobacter* by its inability to oxidize acetate and lactate to CO₂ and water. We propose the following minimal standards for the genus *Gluconobacter*. Cells ellipsoidal to rod shaped; endospores not formed; gram negative or variable. The majority of the strains nonmotile; if motile, the flagellation is polar. Obligately aerobic, possessing a strictly respiratory type of metabolism. Never fermentative. Strong catalase activity. No oxidase. Oxidizes ethanol to acetic acid; rarely very weakly. Does not oxidize acetate or lactate to CO₂ and water. Strong ketogenesis from glycerol. Pronounced acid formation from D-glucose and D-xylose as the pH of the medium drops below 4.5. No acid from or growth on lactose or starch. Produces 2-ketogluconic acid from D-glucose. No nitrate reduction, gelatin liquefaction, indol, or H₂S formation. Optimum temperature, 25 to 30°C. No growth at 37°C.

The occurrence of the phenons A and B raised the question of either (i) splitting the genus *Gluconobacter* into two species or (ii) subdividing *Gluconobacter oxydans* into two subspecies. There are several arguments against splitting. (i) Phenons A and B are differentiated by nicotinic acid requirement only. (ii) A high phenotypic clustering level of at least $S_{SM} = 73\%$ exists between the 98 *Gluconobacter* strains. (iii) Strains belonging to the four subspecies according to *Bergey's Manual* (21) are distributed randomly over both phenotypic and electrophoretic groups A(A') and B(B') (Table 1), and the type strains of the subspecies all cluster in phenon B (Table 1), indicating that the subspecies listed in *Bergey's Manual of Determinative Bacteriology* (21) are not good biological groups. We also examined the position of old pre-1980 species names of *Gluconobacter*, such as "*G. suboxydans*," "*G. roseus*," "*G. dioxyaceticus*," "*G. cerinus*," "*G. albidus*," and a few others in our dendrograms. Strains of each of these species were distributed over both phenons A and B and frequently over several subgroups; they never clustered all together. These old species names do not represent real biological groups and are indeed best forgotten. (iv) The only strain available (IFO 12467^T) of *G. oxydans* subsp. *sphaericus* occupied a slightly separate position in cluster B' (Fig. 2) but not in phenon B (Fig. 1); the position of this strain does not justify separate subspecies status.

We, therefore, advocate a third alternative—that *G. oxydans* (Henneberg 1897) De Ley 1961, 47 remains the only species of the genus *Glu-*

conobacter without further subdivision into subspecies. Phenons A and B are considered here as nicotinic acid-nonrequiring and -requiring biovars, respectively. This opinion is that to be expressed in the next edition of *Bergey's Manual of Determinative Bacteriology*. Strain ATCC 19357^T (= NCIB 9013^T) remains the type strain. For phenon A, the phenotypic centrotpe strain was calculated to be ATCC 15178, and for phenon B, strain NCIB 3734. These calculations were done by the method of Rogers and Tanimoto (43).

Colony variants, twin cultures, and descendant strains. Stable colony variants differing in color, form, and size were examined separately in the following 10 *Gluconobacter* strains: IFO 3251, IFO 3258, IFO 3262, IFO 3267, IFO 3268, IFO 3269, IFO 3285, LMD 29.7, LMD 37.1, and LMD 53.11. Colony variants differed not only in colony characteristics and growth rates but also in some other features. Pairs of colony variants showed from 89 to 95% S_{SM} . For eight strains, the colony variants of the same strain clustered together in the phenotypic and electrophoretic analyses.

We compared electropherograms of twin cultures of several *Gluconobacter* strains from the same original culture which had been maintained by different preservation methods in different culture collections, in some cases for over 40 years. Some cultures of the Institute for Fermentation, Osaka, Japan (IFO) and Laboratorium voor Mikrobiologie, Delft, Netherlands (LMD) had been maintained by serial transfers for years before they were lyophilized. Yet, protein electropherograms of twin cultures of several *Gluconobacter* strains were invariably found to be very similar or identical. For example, (i) the original strain of "*Acetobacter suboxydans*" isolated by Kluver and de Leeuw (36) was maintained at the LMD as strain 23.2^T since 1923, at the National Collection of Type Cultures, London, England (NCTC) since 1944, at the National Collection of Industrial Bacteria, Aberdeen, Scotland (NCIB) as NCIB 7069 since 1949, and in the same collection as NCIB 621^T since 1950; in addition there are a number of descendants such as NCIB 8035, NCIB 8036 (= ATCC 621 H), and NCIB 9498 (Fig. 3). (ii) Strain LMD 29.6 was isolated by J. Frateur in 1929 and maintained since then at LMD and as NCIB 8624 since 1954. (iii) Strain IFO 3262 was isolated by K. Kondô in 1941 and maintained at IFO since 1954 and as LMD 75.3 since 1975. The protein electropherograms of cultures of *Gluconobacter* maintained in our laboratory on GYC medium for 2 years by monthly transfer and their corresponding lyophilized cultures were always identical. Phenotypic testing of the duplicates and descendants of NCIB 621^T

(NCIB 7069, IFO 12528^T, NCIB 8035, and NCIB 9498) revealed an average S_{SM} of 93%, whereas their protein patterns showed an average r of 0.95.

The results obtained by both methods clearly prove the stability of these strains over several decades.

Natural habitats of *Gluconobacter*. The occurrence of *Gluconobacter* in grapes, wine, palm wine, cider, beer, vinegar, fruits, flowers, and bee hives and its role as a causal agent of pink disease of pineapple fruit has been discussed elsewhere (51). *Gluconobacter* has also been isolated from South African sour porridge, baker's yeast, fermenting *Agave* juice, and soft drinks (Table 1). *Gluconobacter* strains might play a role in postharvest losses of fruit by causing rot and browning (57, 58). They have been isolated repeatedly from rotting apples and pears. When injected into the apple varieties Jonared, Golden Delicious, Cox's Orange, and Belle de Boskoop, the isolates induced a necrotic reaction, with the apples showing rot and browning. The apple tissue degraded without soft rot. This phenomenon was accompanied by several shades of browning. Strains were reisolated and proven to be identical to the injected ones by polyacrylamide gel electrophoresis of soluble cell proteins. All *Gluconobacter* strains examined in the present study possessed the capacity to induce rot of apples and pears (58). *Gluconobacter* has been isolated frequently from soft drinks and lemonades (16, 45, 46) and also from bees (38, 44). Lambert et al. (38) showed that two electrophoretically homogeneous groups of strains could be distinguished among isolates from bees in Belgium. One representative strain of each group was included in the present phenotypic study (LMG 79.37 and 79.87). The latter strain is a member of phenon A and the electrophoretic subcluster A'2 (Fig. 3). Strain LMG 79.37, on the other hand, grouped in phenon B and occupied a separate position in the electrophoretic cluster B' (Fig. 2). Most of the A'1 strains were isolated from fruit in Japan, a few from fruit in Belgium, and a few from soft drinks in the Netherlands.

It is also interesting to note that *Gluconobacter* strains isolated in 1941 from flowers in Japan (e.g., IFO 3276), strains isolated in the 1950s from beer in the Netherlands (NCIB 9108 and LMD 53.11), and strains isolated in 1979 from bees in Belgium (e.g., LMG 79.87) belong to the same subcluster A'2 and display almost identical protein electropherograms (Fig. 3), indicating that these strains from very different ecological and geographical niches are genetically highly related.

The strains of subcluster A'3 are of various origins.

Most strains from the electrophoretic subcluster A'4 have been isolated from fruits and flowers in Japan, and two were isolated from rotting pears in Belgium.

Most of the subcluster B'1 and B'2 strains are from beer, and some are from various sources such as fruits in Japan, sour porridge, etc. On the whole, one can say that B strains are mainly beer isolates and that A strains are mainly fruit isolates.

Acetobacter and Frateuria. Both *Acetobacter* and *Frateruia* are phenotypically and electrophoretically quite different from *Gluconobacter* (Fig. 1) (52). Both *Acetobacter aceti* subsp. *liquefaciens* strains examined, IFO 12388^T (= IAM 1834^T) and NCIB 9417, occupy a special position in *Acetobacter* closest to *Gluconobacter* (Fig. 1). In the past, these strains have been named *Gluconobacter liquefaciens* (2, 3, 5, 6, 18). DNA:rRNA hybridizations (24) showed that strain NCIB 9417 is a member of the genus *Acetobacter*. This is further confirmed by its capacity to oxidize acetate and lactate to CO₂ and water. The characteristics of these strains will be discussed in a forthcoming paper on the genus *Acetobacter* (F. Gosselé et al., manuscript in preparation).

ACKNOWLEDGMENTS

J.D.L. is indebted to the Fonds voor Kollektief Fundamenteel Onderzoek for research and personnel grants, J.S. and K.K. to the Nationaal Fonds voor Wetenschappelijk Onderzoek for research grants, and F.G. to the Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw for a scholarship.

We thank I. Banno (IFO), J. van der Toorn (LMD), and G. Kolfsochten for kindly supplying strains and valuable information.

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