Deoxyribonucleic Acid Homologies Among Organisms in the Genus *Gluconobacter*

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The taxonomy of the genus *Gluconobacter* has undergone many changes during the past 40 years. Based on phenotypic properties, this genus has changed from numerous species to one species containing five subspecies and then to a single species, *Gluconobacter oxydans*, with no subspecies. The present study was designed to test the validity of this latter view. Nucleotide sequence similarities were determined for 54 strains of *Gluconobacter* by using an S1 nuclease procedure. Three distinct deoxyribonucleic acid homology groups were obtained. The average level of relatedness among these groups was 16%. Homology group I contained 32 strains and included the type strain of *G. oxydans* and the type strains of all previously recognized subspecies. Homology group II contained 12 strains that had an intragroup homology level of 44 to 87% (average, 65%) with reference strain IFO 3264. Homology group III contained six strains with an average intragroup homology level of 86% with reference strain IFO 3276a. Reference strains IFO 3264 and IFO 3276 were previously recognized as *G. oxydans*. The remaining four strains of *Gluconobacter* had from 0 to 23% homology with reference strains used to delineate the three homology groups. Although these data show that the genus *Gluconobacter* is composed of at least three species, they also support elimination of the previously designated subspecies. Three isolates implicated in pink disease of pineapples were shown either not to be gluconobacters or to be mixed with gluconobacters. The occurrence of colony variants within many of the *Gluconobacter* strains is described, and the significance of this observation is discussed.

The genus *Gluconobacter* is composed of strictly aerobic, gram-negative rods that (i) have the ability to oxidize a wide variety of polyols (17, 25), (ii) lack a complete tricarboxylic acid cycle (21), and (iii) grow at low pH (12). Although these unique features aid in the separation of gluconobacters from other bacteria, the subgeneric taxonomy of this genus has not been clearly established.

Asai (2-5), Vaughn (36), Asai and Shoda (8), and De Ley and Frateur (12) proposed various taxonomic schemes for *Gluconobacter*. All of these proposals were based upon phenotypic data, such as acid production from various carbohydrates, pigmentation, and production of gamma-pyrones from carbohydrates (11, 12). The number of subgeneric taxa proposed by these authors varied from 1 to 13 species (6). In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (12), De Ley and Frateur list a single species, *Gluconobacter oxydans*, containing four subspecies (*G. oxydans* subsp. *industrius*, *G. oxydans* subsp. *melanogenus*, *G. oxydans* subsp. *oxydans*, and *G. oxydans* subsp. *suboxydans*). A fifth subspecies, *G. oxydans* subsp. *sphaericus*, was later described by Ameyama (1).

Loitsyanskaya et al. (27) examined 56 acetic acid bacteria by using 136 phenotypic features and found that all of the *Gluconobacter* strains clustered into a single phenon with a simple matching coefficient of 90%. From this, they concluded that the genus *Gluconobacter* is composed of a single species and that division into separate subspecies is not justified. The same conclusions were reached by Gosselé et al. (20), who investigated 98 strains of *Gluconobacter* and used 177 phenotypic features plus gel electrophoresis of soluble proteins. These conclusions have recently been published in *Bergey's Manual of Systematic Bacteriology*, vol. 1 (14).

The purpose of this investigation was to determine the nucleotide sequence similarity among *Gluconobacter* strains by using an S1 nuclease procedure.

MATERIALS AND METHODS

Bacterial strains. The strains used in the homology experiments and their sources are listed in Table 1. *Escherichia coli* strain B was included in all experiments as a nonhomologous control.

Purity of strains. Prior to use, each strain listed in Table 1 was streaked onto media adjusted to pH 6.0 with HCl and containing 5% sorbitol, 1% yeast extract, 1% peptone, and 1.5% agar (5% sorbitol agar); this was followed by incubation at 28°C for 48 h. Colonies were examined with a dissecting microscope for heterogeneity. If more than one colony type was observed, each type was purified by repeated streakings onto 5% sorbitol agar and designated by placing a lowercase letter after the strain number.

Phenotypic tests. All strains were tested for their ability to oxidize ethanol or lactate to CO₂ and water (33) and for their ability to grow on 10% glucose agar adjusted to pH 4.5 with acetic acid (16).

Culture maintenance. Stock cultures were maintained in 15% glycerol under liquid nitrogen. Working cultures were maintained as unfrozen suspensions in 6 M sorbitol at -15°C (37).

Culture preparation. Subcultures were grown in 50 ml of sorbitol broth (5% sorbitol, 1% yeast extract, and 1% peptone broth, pH 6.0) in 500-ml Bellco nephelometer flasks (37); these preparations were incubated at 28°C and shaken at 400 rpm in a New Brunswick Rotary PsychroTherm incubator. After it reached maximum optical density (in most cases 1.0 U of optical density at 620 nm), the entire 50-ml subculture was inoculated into a 2-liter Erlenmeyer flask containing 500 ml of sorbitol broth and incubated as described above. Usually, 1,500 ml of culture was prepared for each deoxyribonucleic acid (DNA) isolation. Growth

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TABLE 1. Strains used in DNA-DNA homology studies of the genus *Gluconobacter*

Name as received	Culture collection ^a	Strain ^b	Other designation and/or comments ^c
" <i>Acetobacter aurantium</i> "	IFO	IFO 3245 ^{Td}	Type species of <i>Frateruria aurantia</i> (35) ^e
" <i>Acetobacter gluconicus</i> "	UQM	UQM 15	Two colony types ^f
	IFO	IFO 3286 ^{Td}	
" <i>Acetobacter industrius</i> "	ATCC	ATCC 12302	IFO 3260 ^g
	IFO	IFO 3261 ^d	Two colony types, both <i>Gluconobacter</i>
" <i>Acetobacter melanogenus</i> "	ATCC	ATCC 9937 ^d	
" <i>Acetobacter melanogenum</i> "	NRRL	NRRL B58	
" <i>Acetobacter oxydans</i> "	ATCC	ATCC 19357 ^{Td}	Type species of <i>G. oxydans</i> (34); NCIB 9013 ^{Tg}
" <i>Acetobacter rancens</i> "	ATCC	ATCC 23760 ^d	<i>Acetobacter</i>
	IFO	IFO 3297 ^d	Two colony types, both <i>Gluconobacter</i>
" <i>Acetobacter roseus</i> "	ATCC	ATCC 15180	
" <i>Acetobacter species</i> "	CSIRO	CSIRO B1506	
	CSIRO	CSIRO B1507	
" <i>Acetobacter suboxydans</i> "	ATCC	ATCC 621	NCIB 621 ^g
" <i>Acetobacter suboxydans</i> subsp. <i>hoyerium</i> "	C	C 115C	
" <i>Acetobacter viscosus</i> "	ATCC	ATCC 11895 ^d	NCIB 8131 ^g
" <i>Acetobacter xylinum</i> "	CIP	CIP 5714 ^d	
" <i>Gluconobacter albus</i> "	IFO	IFO 3250 ^d	
	IFO	IFO 3251 ^d	
" <i>Gluconobacter capsulatus</i> "	NRRL	NRRL B1225	
" <i>Gluconobacter cerinus</i> "	ATCC	ATCC 19441	IFO 3267 ^g
	IFO	IFO 3264 ^d	
	IFO	IFO 3265 ^d	
	IFO	IFO 3268 ^d	<i>Acetobacter</i>
	IFO	IFO 3270 ^d	
" <i>Gluconobacter dioxyceticus</i> "	IFO	IFO 3271 ^d	
	IFO	IFO 3272 ^d	
" <i>Gluconobacter liquifaciens</i> "	ATCC	ATCC 14835 ^{Td}	IAM 1834 ^T ; <i>Acetobacter liquefaciens</i> (15)
" <i>Gluconobacter melanogenum</i> "	IFO	IFO 3294 ^d	
" <i>Gluconobacter melanogenus</i> "	IFO	IFO 3293 ^d	
" <i>Gluconobacter nonoxygluconicus</i> "	IFO	IFO 3275	Two colony types, both <i>Gluconobacter</i>
	IFO	IFO 3276	Two colony types, both <i>Gluconobacter</i>
" <i>G. oxydans</i> "	ATCC	ATCC 23651 ^d	Two colony types, both <i>Gluconobacter</i> ; NCIB 9014 ^g
	ATCC	ATCC 23652 ^d	NCIB 9119 ^g
	ATCC	ATCC 23771	NCIB 3734 ^g
	ATCC	ATCC 23772	<i>Acetobacter</i> ; NCIB 5595 ^g
	ATCC	ATCC 23775	<i>Acetobacter</i> ; NCIB 8624 ^g
	RS	RS 137	?
	RS	RS 149	?
	RS	RS 203	Possibility of two colony types
	RS	RS 303D	?
<i>G. oxydans</i> subsp. <i>industrius</i>	ATCC	ATCC 33446 ^T	LMD 24.1 ^T ; two colony types, both <i>Gluconobacter</i>
<i>G. oxydans</i> subsp. <i>melanogenus</i>	ATCC	ATCC 33447 ^T	LMD 29.2 ^T
<i>G. oxydans</i> subsp. <i>suboxydans</i>	ATCC	ATCC 33448 ^T	LMD 23.2 ^T
" <i>Gluconobacter roseus</i> "	ATCC	ATCC 14960	NCIB 9137 ^g
	ATCC	ATCC 15178	
" <i>Gluconobacter rubiginosis</i> "	IFO	IFO 3244 ^d	
" <i>Gluconobacter sphaericus</i> "	IFO	IFO 12467 ^T	<i>G. oxydans</i> subsp. <i>sphaericus</i> (34)
" <i>Gluconobacter suboxydans</i> "	ATCC	ATCC 621H	NCIB 8036 ^g
	IFO	IFO 12528 ^d	
" <i>Gluconobacter suboxydans</i> var. <i>al-pha</i> "	IFO	IFO 3254 ^d	
<i>Pseudomonas diminuta</i>	ATCC	ATCC 11568 ^T	Two colony types, both purified
<i>Pseudomonas vesiculare</i>	ATCC	ATCC 11426 ^T	

^a ATCC, American Type Culture Collection, Rockville, Md.; C, Guinness (Dublin) Culture Collection, Dublin, Ireland; CIP, Collection de l'Institut Pasteur, Paris, France; CSIRO, Division of Food Resources, Commonwealth Scientific and Industrial Research Organization, Ryde, Australia; IFO, Culture Collection of the Institute of Fermentation, Osaka, Japan; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; LMD, Laboratorium voor Microbiologie der Landbouwhogeschool, Delft, The Netherlands; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland, United Kingdom; NRRL, Northern Regional Research Center, Peoria, Ill.; RS, pink pineapple disease strains obtained from K. G. Rohrbach, Department of Plant Pathology, University of Hawaii at Manoa, Honolulu, Hawaii; UQM, Department of Microbiology, University of Queensland Medical School, Queensland, Australia.

^b Strains were received from the culture collections indicated unless otherwise noted.

^c Genera were determined as described in the text. Unless otherwise noted, all strains phenotypically appeared to be *Gluconobacter* (Micales, M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1983). ?, Inconclusive results with phenotypic tests for genus.

^d Strain received from N.J. Palleroni, Hoffmann-La Roche Inc., Nutley, N.J.

^e Numbers in parentheses are references.

^f Strain UQM 15a was an *Acetobacter*, and strain UQM 15b was a *Gluconobacter*.

^g Equivalent strain used by Gossele et al. (20).

TABLE 2. Interrelatedness among the reference strains used in homology studies

Unlabeled DNA from:		% Homology with labeled reference DNA from strain: ^a									
Genus	Strain	ATCC 19357 ^T	ATCC 33447 ^T	ATCC 33448 ^T	IFO 12467 ^T	IFO 3264	IFO 3276a	RS 149	ATCC 14835 ^T	IFO 3245 ^T	ATCC 11426 ^T
<i>Gluconobacter</i>	ATCC 19357 ^T	100	76	90	77	18	14	4	2	4	7
	ATCC 33447 ^T	83	100	78	67	10	14	2	7	1	2
	ATCC 33448 ^T	94	78	100	80	11	12	2	8	2	3
	IFO 12467 ^T	65	59	61	100	12	11	3	3	2	1
	IFO 3264	21	19	19	15	100	30	1	0	4	1
	IFO 3276a	14	12	14	10	24	100	2	0	3	1
Unknown	RS 149	4	6	8	4	2	5	100	1	0	3
<i>Acetobacter</i>	ATCC 14835 ^T	10	10	12	8	1	1	4	100	2	2
<i>Frateuria</i>	IFO 3245 ^T	3	5	4	2	5	0	2	2	100	3
<i>Pseudomonas</i>	ATCC 11426 ^T	5	7	7	9	2	0	4	2	0	100

^a All homology values (expressed as percentages of the homologous control) except those obtained with labeled DNA from strain ATCC 11426^T were based on an average of two or more determinations from the same preparation; the values obtained with labeled DNA from strain ATCC 11426^T were based on one determination. The *Gluconobacter* strains used to delimit homology group I were strains ATCC 19357^T, ATCC 33447^T, ATCC 33448^T, and IFO 12467^T, and the strains used to delimit homology groups II and III were strains IFO 3264 and IFO 3276a, respectively.

was followed on undiluted cultures by using a Bausch & Lomb Spectronic 20 spectrophotometer and a 14-mm light path length. Cultures were harvested at 0.8 U of optical density at 620 nm by centrifugation at $16,300 \times g$ for 15 min.

DNA isolation and purification. Cells were suspended in 30 ml of distilled water, and 1.5 ml of 3 M NaCl–0.2 M ethylenediaminetetraacetate (pH 8.0) was added to this suspension. The pH was adjusted to 9.0 with 3 M NaOH, and this was followed by the addition of 0.5 ml of a solution containing 250 U of ribonuclease T₁ per ml and 0.75 ml of a solution containing 0.5 mg of ribonuclease A per ml. Cell walls were partially digested by adding 1 ml of a solution containing 7.5 mg of lysozyme per ml in 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 9.2), followed by shaking for 30 min at room temperature. Lysis was completed by adding 1.5 ml of a 20% sodium dodecyl sulfate solution.

DNA was isolated from bacterial lysates by using a modification of the hydroxylapatite (HA) method described by Johnson (24). After phenol extraction, the cell lysate was dialyzed against a 0.15 M NaCl–0.01 M ethylenediaminetetraacetate solution for 2 h before additional ribonuclease was added to the lysate. After ethanol precipitation and redissolving of the DNA, an additional chloroform extraction step was included; 0.015 M NaCl–0.0015 M trisodium citrate (pH 7.0) was used for all subsequent dialysis and for DNA storage. The HA was prepared by the method of Lachance (26). Prior to use, the HA was boiled in 0.1 M phosphate buffer for 2 to 3 min. The amount of HA was then adjusted to 6 g (wet weight) of HA per 15 ml of HA suspension.

Thermal melting point and purity determinations. The thermal melting points and purities of the isolated DNAs were determined spectrophotometrically (24, 28). The guanine-plus-cytosine (G+C) contents were determined from thermal melting point values by using the formula of Mandel et al. (28). *Escherichia coli* strain B was used as a G+C standard (24). DNA purity was calculated by the method of Johnson (24).

Iodination of reference strain DNA. DNA was labeled with ¹²⁵I by using the method of Selin et al. (32). The average specific activity obtained with this procedure was 1.9×10^6 cpm/μg of DNA. Only DNA preparations that were more than 80% pure were used for reference DNA labeling.

DNA homology experiments. DNA homology values were determined with the free solution S1 nuclease procedure described by Johnson (24). The reassociation mixtures con-

sisted of 50 μl of unlabeled denatured DNA (0.6 mg/ml), 50 μl of 0.88 M NaCl– 10^{-3} M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and 10 μl of labeled denatured DNA and were incubated for 24 h at 68.6°C. Nuclease-resistant duplexes were precipitated as previously described (24), except that 0.5 ml of a 1 N HCl solution containing 1% NaH₂PO₄ and 1% Na₄P₂O₇ (29) was used. The DNA precipitates were collected on Whatman GF/F glass fiber filter strips by using a 1:4 dilution of the acid solution and dried under a heat lamp for 1 h, and the radioactivity was measured with a Beckman model 5500 gamma counter.

RESULTS

The levels of purity of the DNA preparations used in this study ranged from 59 to 99% (average, 77%).

Reciprocal homology values for the reference strains used in this study are shown in Table 2. The levels of homology among the type strains of four of the five *G. oxydans* subspecies (34) ranged from 59 to 94%. Reference strains representing two additional groups of *Gluconobacter* (strains IFO 3264 and IFO 3276a) had levels of homology with the *G. oxydans* strains that ranged from 10 to 21%. The remaining reference strains in Table 2 represent genera that are phenotypically similar to *Gluconobacter*; these strains had levels of homology of ≤10% with the *Gluconobacter* strains.

DNA preparations from 59 organisms were compared with the reference organisms; 32 of these strains were placed in homology group I and had G+C contents that ranged from 54 to 59 mol% (Table 3). These strains had 34 to 99% homology with the four *Gluconobacter* type strains. Compared with the type species (strain ATCC 19357^T [T = type strain]), 27 strains had levels of homology that were greater than 60% (average, 86%). Three of the remaining four strains demonstrated more than 50% homology with the type species, *G. oxydans* (strain ATCC 19357^T).

Homology group II consisted of 12 strains which had G+C contents that ranged from 53 to 55 mol% (Table 4). Compared with reference strain IFO 3264, the levels of homology ranged from 44 to 87% (average, 65%).

Six strains belonged to homology group III, which was delineated with reference strain IFO 3276a (Table 4). The G+C contents ranged from 52 to 55 mol%. Strain IFO 3276b had 99% homology with strain IFO 3276a; thus, we concluded that these two isolates were colony variants of the same strain. The levels of homology with the five remaining

TABLE 3. Homology group I: DNA homology values of strains having high levels of homology with *Gluconobacter* type strains

Source of unlabeled DNA	G+C content (mol%)	% Homology with labeled reference DNA from strain:			
		ATCC 19357 ^T	ATCC 33447 ^T	ATCC 33448 ^T	IFO 12467 ^T
ATCC 19357 ^T	59	100	76	90	77
NRRL B1225	ND ^a	99	76	92	75
ATCC 11895	ND	96	73	90	76
IFO 12528	ND	95	76	94	72
ATCC 621H	ND	94	76	92	77
ATCC 33448 ^T	59	94	78	100	80
C 115C	ND	93	75	86	74
CIP 5714	ND	93	76	92	72
ATCC 23652	ND	92	76	100	79
ATCC 33446 ^T	59	92	74	87	83
ATCC 33446a ^b	57	80	76	87	72
ATCC 33446b	59	87	77	85	79
IFO 3261a ^c	59	92	79	93	70
ATCC 621	ND	91	73	89	82
ATCC 23771	55	90	74	94	69
CSIRO B1506	ND	87	90	83	73
IFO 3244	ND	86	80	83	62
ATCC 9937	ND	85	75	82	75
IFO 3294	ND	85	81	83	67
ATCC 33447 ^T	56	83	100	78	67
CSIRO B1507	ND	83	82	82	70
IFO 3293	ND	83	84	83	52
IFO 3297b ^c	58	83	82	82	70
ATCC 23760	ND	81	77	79	70
ATCC 23772	ND	81	71	81	65
NRRL 58	ND	76	75	75	67
IFO 12467	57	65	59	61	100
ATCC 14960	56	61	57	54	54
IFO 3250	59	53	46	48	52
ATCC 23651a	57	51	38	53	41
ATCC 23651b	57	52	40	49	40
IFO 3275a ^c	54	42	39	41	34

^a ND, Not determined.^b Lowercase letters after a strain number indicate different colony types isolated from the original strain.^c Strain having two colony types which are in separate homology groups.

strains in homology group III ranged from 71 to 99% (average, 86%).

Table 5 shows results for four additional strains exhibiting phenotypic properties (Table 1) of acetic acid bacteria (7, 11). Strains ATCC 19441 and ATCC 23775 had significant levels of homology (19 and 20%) with the type species, *G. oxydans* (strain ATCC 19357^T), whereas strains IFO 3261b and UQM 15b showed about 10% homology with the type species (Table 5).

Strains RS 137, RS 149, and RS 303D reportedly cause pink disease of pineapples (K. G. Rohrbach, personal communication). The G+C contents of these strains ranged from 50 to 53 mol% (Table 5). These three Rohrbach strains showed a high degree of homology (>90%) with strain RS 149 (Table 5). In addition, strain RS 303D had significant levels of homology with strains IFO 3264 and IFO 3276a (Table 5).

DISCUSSION

Differentiation of *Gluconobacter* from other genera. De Ley et al. (13) placed *Gluconobacter* and *Acetobacter* in the family *Acetobacteraceae*. These two genera are commonly referred to as the acetic acid bacteria, and they are composed of gram-negative or gram-variable, ellipsoidal to rod-shaped cells that (i) have a strictly respiratory type of

metabolism with oxygen as the terminal electron acceptor, (ii) are oxidase negative, (iii) oxidize ethanol to acetic acid, (iv) grow in acidic media, and (v) are found in sugary and alcoholized, slightly acid niches, such as fruits, flowers, fermented plant juices, and honey. Primary phenotypic differentiation between these two genera depends upon the fact that the acetobacters have a functional tricarboxylic acid cycle (15) and the gluconobacters do not (14). The presence or absence of a functional tricarboxylic acid cycle is determined by a test that shows complete oxidation of ethanol or lactate to CO₂ and water during growth on solid media (16).

The genus *Frateruria* resembles *Gluconobacter* in a number of respects, most notably in its ability to oxidize ethanol to acetic acid and grow under acidic conditions (35). Unlike the gluconobacters, however, the fraterurias completely oxidize lactate to CO₂ and water, produce H₂S, and grow in the absence of vitamins (35).

Isolation and characterization of gluconobacters commonly involves growth on an acidic medium (9, 14), but the recent literature does not indicate which acid should be used to lower the pH of the isolation medium. In 1950, Frateur (16) recommended acidifying isolation media with acetic acid and then culturing the bacteria on a medium containing 10% sugar. We have found that a medium containing 10% glucose, 1% yeast extract, and 1% peptone works well for isolating and growing acetic acid bacteria when acetic acid is used to adjust the pH to 4.5 (C. A. Baker and G. W. Claus, unpublished data). However, we also have found that many other bacteria, including *E. coli*, grow if HCl is used to acidify this medium instead of acetic acid (Micales, unpublished data). Our observations suggest that it is acetate anion tolerance, not just acidic conditions, that differentiates acetic acid bacteria and fraterurias from other bacteria. We

TABLE 4. Homology groups II and III: strains having high levels of homology with *Gluconobacter* reference strains IFO 3264 and IFO 3276a

Source of unlabeled DNA	G+C content (mol %)	% Homology with labeled reference DNA from strain:	
		IFO 3264	IFO 3276a ^a
Homology group II			
IFO 3264	55	100	30
IFO 3272	55	87	32
IFO 3271	55	77	26
UQM 15a ^b	53	75	23
ATCC 12302	55	70	24
ATCC 15178	55	68	25
IFO 3270	55	64	29
ATCC 15180	55	62	24
IFO 3251	55	59	ND ^c
IFO 3286	55	58	35
IFO 3268	54	54	25
IFO 3254	55	44	20
Homology group III			
IFO 3276a	54	22	100
IFO 3276b	54	23	99
IFO 3265	55	65	99
IFO 3275b ^b	52	67	97
IFO 3297a ^b	54	21	77
RS 203b	53	19	71

^a Lowercase letters after a strain number indicate different colony types isolated from the original strain.^b Strain having two colony types which are in separate homology groups.^c ND, Not determined.

TABLE 5. DNA homology values for other acetic acid bacteria and Rohrbach strains

Source of unlabeled DNA	G+C content (mol %)	% Homology with labeled reference DNA from strain: ^a									
		ATCC 19357 ^T	ATCC 33447 ^T	ATCC 33448 ^T	IFO 12467 ^T	IFO 3264	IFO 3276a ^b	RS 149	ATCC 14835 ^T	IFO 3245 ^T	ATCC 11426 ^T
ATCC 19441	55	20	17	21	14	23	ND ^c	ND	0	0	ND
ATCC 23775	55	19	18	19	17	11	ND	ND	3	4	ND
IFO 3261b ^d	59	10	11	13	6	0	0	2	2	0	0
UQM 15b ^d	53	10	10	9	14	0	3	3	0	1	2
RS 137 ^e	51	7	9	9	11	ND	ND	92	1	1	ND
RS 149 ^e	50	4	6	8	4	2	5	100	1	0	3
RS 303D ^e	51	20	21	21	22	41	68	93	2	4	1

^a All homology values except those obtained with labeled DNA from strain ATCC 11426^T were based on an average of two or more determinations from the same preparation; the values obtained with labeled DNA from strain ATCC 11426^T were based on one determination.

^b Lowercase letters after a strain number indicate different colony types isolated from the original strain.

^c ND, Not determined.

^d Strain having two colony types which are in separate homology groups.

^e Rohrbach strains RS 137, RS 149, and RS 303D reportedly cause pink disease of pineapples. These strains phenotypically did not appear to be acetic acid bacteria, nor did they fit well into any of the *Gluconobacter* homology groups.

suspect that some workers have failed to use acetic acid to lower the pH of isolation media and that this has contributed to the misidentification of certain gram-negative, aerobic, rod-shaped isolates as acetic acid bacteria.

Subgeneric status. Recent phenotypic studies (8, 11, 12, 14, 19, 20, 27) suggest that the genus *Gluconobacter* should be composed of one species, *G. oxydans*. Although unpublished DNA homology results of Gillis (20) indicate genetic heterogeneity among *G. oxydans* strains, our more comprehensive study shows three distinct homology groups within this genus (Tables 3 and 4) with an average intergroup DNA homology level of 16% (Table 2). Considering the proposal of Johnson (24) coupled with the procedures correlation study by Grimont et al. (22), our data support the proposal that each *Gluconobacter* homology group be considered a separate species. Since the type strain of *G. oxydans* is in homology group I, we presently regard all homology group I strains as *G. oxydans*. We also regard homology groups II and III as separate, but as yet unnamed, species of *Gluconobacter*.

The recent phenotypic studies cited in the preceding paragraph suggest that subspecies designations within this genus be eliminated. Our data also support this elimination, since all type strains belonged to the same homology group (Table 2). We recognize a certain degree of heterogeneity within these three homology groups, but we do not recommend additional subgrouping at this time because of the small number of strains tested and a lack of obvious phenotypic differences.

Comparison with previous numerical taxonomy data. Gosselé et al. (20) used phenotypic tests and gel electrophoresis to examine 98 *Gluconobacter* strains, and they concluded that *Gluconobacter* contains one species, *G. oxydans*, which should not be divided into subspecies. These authors found some variation within the genus, which grouped their strains into two phenon; phenon B strains required nicotinic acid, and phenon A strains did not. They also observed slight differences in protein electrophoresis patterns.

In the present study we included 29 of the strains examined by Gosselé et al. (20). All 15 of the phenon B strains were in our homology group I (Table 6). Of the 14 phenon A strains, 1 was in homology group I, 9 were in homology group II, and 2 each were in homology group III and the unidentified group. Since all but one of the 16 homology group I strains (*G. oxydans*) used in both studies belonged to phenon B, it appears that the requirement for nicotinic acid

is a valuable characteristic for differentiating this species from other *gluconobacters*.

Rohrbach strains. Bacterial pink disease of pineapple fruit is a condition previously attributed to certain strains of *Gluconobacter oxydans* (30, 31), such as strains RS 137, RS 149, RS 203, and RS 303D (K. G. Rohrbach, personal

TABLE 6. Comparison of DNA homology groups from the present study and phenon described by Gosselé et al.

Homology group ^a	Strain ^b	Phenon of Gosselé et al. ^c
I	ATCC 621	B
	ATCC 621H	B
	ATCC 11895	B
	ATCC 14960	B
	ATCC 19357	B
	ATCC 23651a,b	B
	ATCC 23652	B
	ATCC 23771	B
	ATCC 23772	B
	ATCC 33446a,b	B
	ATCC 33447	B
	IFO 3244	B
	IFO 3293	B
	IFO 3294	A
	IFO 12467	B
II	IFO 12528	B
	ATCC 12302	A
	ATCC 15178	A
	ATCC 15180	A
	IFO 3251	A
	IFO 3264	A
	IFO 3268	A
	IFO 3270	A
	IFO 3271	A
	IFO 3286	A
III	IFO 3265	A
	IFO 3276a,b	A
Unidentified	ATCC 19441	A
	ATCC 23775	A

^a The homology groups are those shown in Tables 3 and 4. Levels of homology of unidentified strains are shown in Table 5.

^b The strains used in this study are the same as those used by Gosselé et al. (20) according to the information in culture collection catalogs. Lowercase letters after strain numbers indicate colony types isolated during this investigation.

^c See reference 20.

communication). However, our DNA homology studies showed that only strain RS 203b, one of two colony types isolated from the organism received as strain RS 203, was a *Gluconobacter*. Colony type RS 203b had 71% homology with the homology group III reference strain (Table 4), and it exhibited typical phenotypic characteristics of *Gluconobacter*. The other colony type, colony type RS 203a, was lost during storage.

Although the remaining Rohrbach strains (strains RS 137, RS 149, and RS 303D) are closely related to each other, they do not appear to be strains of *Gluconobacter*, *Acetobacter*, or *Frateuria*, because they did not grow on 10% glucose agar at pH 4.5 and they did not completely oxidize ethanol or lactate. Although strain RS 303D demonstrated only one colony type, our DNA homology values suggested that it was a mixed culture (Table 5). Strain RS 303D had 93% homology with strain RS 149, 68% homology with the homology group II indicator strain (IFO 3264), and 41% homology with the homology group III indicator strain (IFO 3276a). This may explain why this strain was previously identified by others as a *Gluconobacter* species. Our results with the Rohrbach strains prompt us to caution others in attributing pineapple phytopathogenicity to the gluconobacters.

Colony variants and culture purity. Although most of the cultures used in this study were pure, some exhibited two or more stable colony types (Table 1). These were often difficult to detect without using a dissecting microscope. In some cases, separate colony types isolated from a single culture showed almost identical homology values when they were compared with reference strains (e.g., colony types ATCC 23651a and ATCC 23651b, ATCC 33446a and ATCC 33446b, and IFO 3276a and IFO 3276b) (Tables 3 and 4). With other cultures, however, separate colony types were found to be in different homology groups (e.g., colony types IFO 3261a and IFO 3261b, IFO 3275a and IFO 3275b, and IFO 3297a and IFO 3297b) (Tables 3 through 5).

Multiple colony types from other cultures did not give such clear-cut results. For example, strain IFO 3261 was found by ribosomal ribonucleic acid homology to be an *Acetobacter* (18). Similarly, T. Iijima and K. Imai (Institute for Fermentation, Osaka, Japan, personal communication) found that this strain completely oxidizes lactate to CO₂ and water, which also places it in the genus *Acetobacter*. We found that strain IFO 3261 had two colony types; colony type IFO 3261a was a *G. oxydans* strain (homology group I); and colony type IFO 3261b phenotypically appeared to be a *Gluconobacter* (Table 1), but it had only low levels of homology with the *G. oxydans* reference strains (Table 5). Although our results do not support the hypothesis that strain IFO 3261 is an *Acetobacter*, the taxonomic position of colony type IFO 3261b still is not clear.

Another interesting example was strain UQM 15. Phenotypically, colony type UQM 15a was an *Acetobacter* (Table 1); however, it had 75% homology with the *Gluconobacter* homology group II reference strain. Thus, colony type UQM 15a appeared to be a phenotypically unusual *Gluconobacter*. Colony type UQM 15b, like colony type IFO 3261b, demonstrated typical *Gluconobacter* phenotypic characteristics and also showed about 10% homology with the *G. oxydans* reference strains (Table 5). Thus, colony types UQM 15b and IFO 3261b appear to be similar and may represent another *Gluconobacter* species.

On the other hand, mixed cultures may not always be evident by examining colony morphology. For example, strain IFO 3265 and colony type IFO 3275b each contained

only one colony type, yet each culture showed high levels of homology with both homology group II and homology group III strains (Table 4). Therefore, strain IFO 3265 and colony type IFO 3275b may each be a mixture of two *Gluconobacter* species that exhibit identical colony types.

The reason(s) for the common occurrence of mixed cultures of acetic acid bacteria is not clear. Spontaneous mutation at a high frequency has been considered (10), but there seems to be little evidence to support this idea. We have observed that cells in *Gluconobacter* or *Acetobacter* cultures often stick tightly together in clumps of micro- or macroscopic size. This cell-to-cell adhesion appears to be influenced by both medium composition and growth phase. It seems likely that the ability of acetic acid bacteria to stick to one another causes problems in culture purification. For example, if cells from one type of *Gluconobacter* are present in very low numbers and are tightly stuck to clumps of a more predominant yet genetically different type, the presence of this minor colony type may not be detected on streak plates. However, if some physical or chemical change in the environment selectively causes the minor colony type to become more numerous, or if an environmental change causes clumps to be dispersed so that cells from the minor colony type are released, then different colony types might seem to appear spontaneously. This latter reasoning may explain why we recently recovered a mixture of two colony types from a culture whose purity was rigorously checked prior to storage for several years in liquid nitrogen.

We wonder whether undetected mixtures of acetic acid bacteria have caused other workers to describe strains that are "intermediate" between *Gluconobacter* and *Acetobacter* (7). It is also conceivable that shifts in proportions of different colony types may have been responsible for the phenotypic variations observed in some strains of acetic acid bacteria (6-8).

The tendency for cells to adhere to one another makes these cultures difficult to work with by using classical pure culture techniques. Our experience with cultures from other culture collections suggests that other workers may also be working with mixed colony types of acetic acid bacteria. To avoid continuance of this understandable problem, we recommend that investigators (i) use a dissecting microscope to examine many well-isolated colonies from a number of streak or spread plates before homogeneity is assumed, and (ii) prepare each new stock culture from a single, well-isolated colony. Although application of these procedures should reduce the number of mixed *Gluconobacter* cultures, it will not prevent investigators from working with different *Gluconobacter* species that have identical colony types. Perhaps a comparison of DNA homology with a reference strain of indisputable purity is the only reliable criterion that a *Gluconobacter* or *Acetobacter* culture is pure.

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LITERATURE CITED

- Ameyama, M. 1975. *Gluconobacter oxydans* subspecies *sphaericus*, new subspecies isolated from grapes. *Int. J. Syst. Bacteriol.* **23**:365-370.
- Asai, T. 1935. A systematic study of alcohol- and carbohydrate-oxidizing bacteria isolated from fruits and a new classification of oxidizing bacteria III. *Nippon Nogei Kagaku Kaishi* **11**:674-708.
- Asai, T. 1935. A systematic study of alcohol- and carbohydrate-oxidizing bacteria isolated from fruits and a new classification of oxidizing bacteria III. *Nippon Nogei Kagaku Kaishi* **11**:610-620.
- Asai, T. 1935. A systematic study of alcohol- and carbohydrate-oxidizing bacteria isolated from fruits and a new classification of oxidizing bacteria III. *Nippon Nogei Kagaku Kaishi* **11**:499-513.
- Asai, T. 1935. A systematic study of alcohol- and carbohydrate-oxidizing bacteria isolated from fruits and a new classification of oxidizing bacteria III. *Nippon Nogei Kagaku Kaishi* **11**:377-390.
- Asai, T. 1968. *Acetic acid bacteria*. University of Tokyo Press, Tokyo.
- Asai, T., H. Iizuka, and K. Komagata. 1964. The flagellation and taxonomy of genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains. *J. Gen. Appl. Microbiol.* **10**:95-126.
- Asai, T., and K. Shoda. 1958. The taxonomy of *Acetobacter* and allied oxidative bacteria. *J. Gen. Appl. Microbiol.* **4**:289-311.
- Carr, J. G., and S. M. Passmore. 1979. Methods for identifying acetic acid bacteria, p. 33-47. In F. A. Skinner and D. W. Lovelock (ed.), *Identification methods for microbiologists*, 2nd ed. Academic Press, Inc., London.
- Carr, J. G., and J. L. Shimwell. 1961. The acetic acid bacteria, 1941-1961: a critical review. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **27**:386-400.
- De Ley, J. 1961. Comparative carbohydrate metabolism and a proposal for the phylogenetic relationship of the acetic acid bacteria. *J. Gen. Microbiol.* **24**:31-50.
- De Ley, J., and J. Frateur. 1974. *Gluconobacter* Asai 1935, p. 251-253. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams & Wilkins Co., Baltimore.
- De Ley, J., M. Gillis, and J. Swings. 1984. Family VI. *Acetobacteraceae* Gillis and De Ley 1980, p. 267-268. In N. R. Krieg (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- De Ley, J., and J. Swings. 1984. The genus *Gluconobacter* Asai 1935, emend. mut. char. Asai, Iizuka, and Komagata 1964, p. 275-278. In N. R. Krieg (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- De Ley, J., J. Swings, and F. Gosselé. 1984. Genus 1. *Acetobacter* Beijerinck 1898, 215, p. 268-274. In N. R. Krieg (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- Fratureur, J. 1950. Essai sur la systématique des acetobacters. *Cellule* **53**:287-392.
- Fulmer, E. I., and L. A. Underkofler. 1947. Oxidation of polyhydric alcohols by *Acetobacter suboxydans*. *Iowa State Coll. J. Sci.* **21**:251-270.
- Gillis, M., and J. De Ley. 1980. Intra- and intergeneric similarities of the ribosomal ribonucleic acid cistrons of *Acetobacter* and *Gluconobacter*. *Int. J. Syst. Bacteriol.* **30**:7-27.
- Gosselé, F., J. Swings, and J. De Ley. 1980. Growth factor requirements of *Gluconobacter*. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* **1**:348-350.
- Gosselé, F., J. Swings, K. Kersters, and J. De Ley. 1983. Numerical analysis of phenotypic features and protein gel electropherograms of *Gluconobacter* Asai 1935 emend. mut. char. Asai, Iizuka, and Komagata 1964. *Int. J. Syst. Bacteriol.* **33**:65-81.
- Greenfield, S., and G. W. Claus. 1972. Nonfunctional tri-carboxylic acid cycle and the mechanism of glutamate biosynthesis in *Acetobacter suboxydans*. *J. Bacteriol.* **112**:1295-1301.
- Grimont, P. A., M. Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin. 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr. Microbiol.* **4**:325-330.
- Johnson, J. L. 1973. Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. *Int. J. Syst. Bacteriol.* **23**:308-315.
- Johnson, J. L. 1981. Genetic characterization, p. 450-472. In P. Gehardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, and W. A. Wood (ed.), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D.C.
- Kersters, K., and J. De Ley. 1963. The oxidation of glycols by acetic acid bacteria. *Biochim. Biophys. Acta* **71**:311-331.
- Lachance, M. 1980. Simple method for determination of deoxyribonucleic acid relatedness by thermal elution in hydroxyapatite microcolumns. *Int. J. Syst. Bacteriol.* **30**:433-436.
- Loitsyanskaya, M. S., G. V. Pavlenko, and A. I. Ivchenko. 1979. Taxonomy of acetic acid bacteria. *Mikrobiologiya (Engl. Transl.)* **48**:545-551.
- Mandel, M., L. Igambi, J. Bergendahl, M. L. Dodson, Jr., and E. Scheltgen. 1970. Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acid. *J. Bacteriol.* **101**:333-338.
- Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237-251.
- Rohrbach, K. G., and J. B. Pfeiffer. 1975. The field induction of bacterial pink disease in pineapple fruit. *Phytopathology* **65**:803-805.
- Rohrbach, K. G., and J. B. Pfeiffer. 1976. The interaction of four bacteria causing pink disease of pineapple with several pineapple cultivars. *Phytopathology* **66**:396-399.
- Selin, Y. M., B. Harich, and J. L. Johnson. 1983. Preparation of labeled nucleic acids (nick translation and iodination) for DNA homology and rRNA hybridization experiments. *Curr. Microbiol.* **8**:127-132.
- Shimwell, J. L., J. G. Carr, and M. E. Rhodes. 1960. Differentiation of *Acetomonas* and *Pseudomonas*. *J. Gen. Microbiol.* **23**:283-286.
- Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (ed.). 1980. Approved lists of bacterial names. *Int. J. Syst. Bacteriol.* **30**:225-420.
- Swings, J., M. Gillis, K. Kersters, P. DeVos, F. Gosselé, and J. De Ley. 1980. *Fratureia*, a new genus for *Acetobacter aurantius*. *Int. J. Syst. Bacteriol.* **30**:547-556.
- Vaughn, R. H. 1942. The acetic acid bacteria. *Wallerstein Lab. Commun.* **5**:6:5-26.
- White, S. A., and G. W. Claus. 1982. Effect of intracytoplasmic membrane development on oxidation of sorbitol and other polyols by *Gluconobacter oxydans*. *J. Bacteriol.* **150**:934-943.