Taxonomic heterogeneity of strains comprising *Gluconacetobacter hansenii*

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The taxonomic standing of *Gluconacetobacter hansenii* was clarified through phenotypic characteristics, quinones, DNA base composition, DNA relatedness, and the production of gluconic and ketogluconic acids from glucose. All strains that Gosselé et al. (*Syst. Appl. Microbiol.*, 4, 338–368, 1983) employed in the establishment of *Acetobacter hansenii* (= *G. hansenii*) were used in this study. Phenotypic differences were shown among the strains of *G. hansenii*, suggesting heterogeneity within the species. The major ubiquinone was Q-10 for all strains of *G. hansenii*, except for strain IFO 3296, which was characterized by Q-9. This excluded IFO 3296 from the species *G. hansenii* and placed it in the genus *Acetobacter*. DNA relatedness revealed four distinct homology groups (I, II, III, and IV) among strains of the species. Group I was distinguished from the other genomic groups by a lower G+C range from 58.9 to 59.2 mol%. Groups II, III, and IV showed higher G+C contents of 60.4 to 62.2, 60.8, and 61.7 mol%, respectively. Groups I and IV produced both 2- and 5-ketogluconic acids from glucose, and Group III produced only 2-ketogluconic acid. Group II included strains that produced both 2- and 5-ketogluconic acids and strains that produced only 2-ketogluconic acid. It is clear that *G. hansenii* consists of genotypically heterogeneous strains comprising four homology groups (I, II, III, and IV). Since group I contains the type strain (IFO 14820^T=LMG 1527^T) of the species, this group is designated as the species *G. hansenii*.

Key Words—*Acetobacter*; *Gluconacetobacter*; *Gluconacetobacter hansenii*

The species *Acetobacter hansenii* was established on the basis of numerical phenotypic analysis and protein electrophoregrams for subphenon C of phenon 4 by Gosselé et al. (1983). However, quinones and DNA relatedness between strains used were not taken into account in establishing the species. The current practice defines species in phylogenetic terms and regards DNA reassociation as the sequence standard (Wayne et al., 1987). Furthermore, the range of DNA base composition of the *A. hansenii* strains was broad, 4.5 mol% (Gosselé et al., 1983). The strains of this species originated from seven strains of *Acetobacter aceti* subsp. *xylinum*, three strains of *A. aceti* subsp. *orleanensis*, and one strain of *A. pasteurianus* subsp. *pasteurianus* (= *A. pasteurianus*). Of the seven

strains of *Acetobacter aceti* subsp. *xylinum*, one was received as *Gluconobacter oxydans*.

Yamada et al. (1997) proposed the 4-genus concept of acetic acid bacteria, i.e., *Acetobacter*, *Gluconoacetobacter* (*sic*), *Acidomonas*, and *Gluconobacter*, on the basis of partial sequences of 16S rRNA. The name *Gluconoacetobacter* (*sic*) was later corrected to *Gluconacetobacter* (ICSB, 1998). Consequently, *Acetobacter hansenii*, *A. liquefaciens*, *A. xylinum*, *A. diazotrophicus*, and *A. europaeus* were included in the genus *Gluconacetobacter* with the following changes in nomenclature: *G. hansenii*, *G. liquefaciens*, *G. xylinus*, *G. diazotrophicus*, and *G. europaeus*.

This paper intends to elucidate the taxonomic standing of *G. hansenii* by using the same strains employed by Gosselé et al. (1983), based on phenotypic characteristics, quinones, DNA base composition, DNA relatedness, and the production of gluconic and ketogluconic acids from glucose.

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Table 1. *Gluconacetobacter hansenii* strains studied, citing the strain designations used by Gosselé et al. (1983).

Strain No.	Strain number and strain designation used by Gosselé et al. (1983)	
IFO 14820 ^T	NCIB 8746 ^T	<i>A. aceti</i> subsp. <i>orleanensis</i>
LMG 1527 ^T	NCIB 8746 ^T	<i>A. aceti</i> subsp. <i>orleanensis</i>
IFO 14817	NCIB 8747	<i>A. aceti</i> subsp. <i>orleanensis</i>
LMG 1582	LMD 29.3	<i>A. aceti</i> subsp. <i>orleanensis</i>
IFO 14815	NCIB 8752	<i>A. aceti</i> subsp. <i>xylinum</i>
IFO 14816	NCIB 8246	<i>A. aceti</i> subsp. <i>xylinum</i>
IFO 14822	NCIB 4940	<i>A. aceti</i> subsp. <i>xylinum</i>
LMG 1517	NCIB 4940	<i>A. aceti</i> subsp. <i>xylinum</i>
LMG 1536	Nata	<i>A. aceti</i> subsp. <i>xylinum</i>
LMG 1688	LMD 22.3	<i>A. aceti</i> subsp. <i>xylinum</i>
LMG 1689	LMD 29.8	<i>A. aceti</i> subsp. <i>xylinum</i>
IFO 3261 ^a	IFO 3261	<i>A. aceti</i> subsp. <i>xylinum</i>
IFO 3296	IFO 3296	<i>A. pasteurianus</i> subsp. <i>pasteurianus</i>

^a Received as *Gluconobacter oxydans*.^T Type strain.

Materials and Methods

Bacterial strains. The strains used are listed in Table 1. They were checked for identity and purity before use, as described previously (Navarro and Komagata, 1999).

Phenotypic characterization, quinone analysis, DNA extraction, determination of DNA base composition (mol% G+C), DNA-DNA hybridization, and determination of gluconic acid and ketogluconic acid production from glucose. Methods were mostly those described previously (Navarro and Komagata, 1999). DNA-DNA hybridization followed the procedure of Ezaki et al. (1989). Hybridization temperatures differed among probes, but were in a range from 48.4 to 49.8°C. The duration of hybridization was 16 h. Growth on ethanol was determined by growing cultures in the Hoyer-Frateur medium composed of 0.1% (NH₄)₂SO₄, 0.01% K₂HPO₄, 0.09% KH₂PO₄, 0.025% MgSO₄·7H₂O, 0.0005% FeCl₃·6H₂O, and 3% ethanol (De Ley and Frateur, 1974). Growth was observed after 3 days incubation at 30°C. The production of cellulose was confirmed by boiling the pellicle in 5% NaOH. Cellulose was confirmed if the pellicle did not dissolve after 2 h of boiling.

Results

Phenotypic characteristics

Strains of *G. hansenii* were Gram-negative and rod shaped. No strains showed motility. All strains oxidized ethanol to acetic acid, and with the exception of IFO 3261, overoxidized acetate and lactate to CO₂ and H₂O. All were catalase-positive and oxidase-negative. They grew on glutamate and mannitol agar, but

Table 2. Phenotypic characteristics of *Gluconacetobacter hansenii* strains.

Strain No.	Motility	Oxidation of			Production of			Ketogenesis from glycerol	Assimilation of ammoniacal N ₂	Growth on ethanol	Growth on glutamate agar	Growth on mannitol agar	γ-Pyrrone production from	
		Ethanol	Acetate	Lactate	Oxidase	Catalase	Cellulose						Glucose	Fructose
IFO 14820 ^T	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LMG 1527 ^T	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IFO 14817	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LMG 1582	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IFO 14815	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IFO 14816	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IFO 14822	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LMG 1517	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LMG 1536	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LMG 1688	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LMG 1689	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IFO 3261	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IFO 3296	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Note: All strains were Gram-negative and rod shaped.

^T Type strain.

Table 3. Ubiquinones of *Gluconacetobacter hansenii* strains.

Strain No.	Ubiquinone (%)		
	Q-8	Q-9	Q-10
IFO 14820 ^T	—	16	84
LMG 1527 ^T	—	12	88
IFO 14817	—	6	94
LMG 1582	—	10	90
IFO 14815	—	7	93
IFO 14822	—	3	97
LMG 1517	—	9	91
LMG 1688	—	5	95
LMG 1689	3	3	94
IFO 3261	—	9	91
IFO 3296	—	84	16

Note: The quinone systems of strains IFO 14816 and LMG 1536 were not determined because of difficulty in quinone extraction posed by the extreme production of cellulose.

^T Type strain.

did not grow on ethanol as a sole carbon source. All strains, except IFO 3296, produced dihydroxyacetone from glycerol. Of the 11 strains, IFO 14822 (=LMG 1517), IFO 14815, IFO 14816, and LMG 1536 produced cellulose pellicles. Strains IFO 14820^T (=LMG 1527^T), IFO 14817, LMG 1582, and IFO 3296 assimilated ammoniacal nitrogen. No strains produced γ -pyrones from glucose; only IFO 14815 produced γ -pyrones from fructose. The data are shown in Table 2.

Ubiquinone system

The *G. hansenii* strains tested had Q-10 as the major ubiquinone, with the exception of IFO 3296 (Table 3). IFO 3296 had Q-9.

DNA base composition

The range of DNA base composition of strains of *G. hansenii* was from 58.9 to 62.2 mol%, as shown in Table 4.

DNA relatedness

Four homology groups (I, II, III, and IV) were delineated among strains of *G. hansenii* (Table 4). Homology group I included the type strain of *G. hansenii*, IFO 14820^T (=LMG 1527^T) and IFO 14817, both formerly designated as *A. aceti* subsp. *orleanensis*. Homology group II included four strains formerly identified as *A. aceti* subsp. *xylinum* (IFO 14822 [=LMG 1517], LMG 1688, LMG 1689, and IFO 3261). Groups III and IV contained one strain each, namely, LMG 1582 (former *A. aceti* subsp. *orleanensis* strain) and IFO 14815 (former *A. aceti* subsp. *xylinum* strain), respectively. The levels of DNA relatedness between strains within a homology group were high, ranging from 83 to 116% for group I and from 57 to 112% for

group II. On the other hand, those between strains of groups I, II, and III were low, ranging from 8 to 49%. Group IV showed high levels (27 to 59%) of DNA relatedness with other groups. However, the values were less than 70%. Therefore we recognized it as an independent group.

All strains of *G. hansenii* had low levels of DNA relatedness with other species of acetic acid bacteria, namely, *G. xylinus* LMG 1515^T (1 to 10%), *G. liquefaciens* AJ 12950^T (5 to 19%), *G. diazotrophicus* NCIMB 12985 (6 to 19%), *Acetobacter aceti* IFO 14818^T (6 to 20%), *A. pasteurianus* LMG 1262^T (11 to 43%), and *Gluconobacter oxydans* (15 to 41%). The data are shown in Table 5. The same low levels of DNA-DNA homology (2 to 9%) were observed in strains of group I when *A. pasteurianus* IFO 13752 (the type strain of former *A. aceti* subsp. *orleanensis*) was used as a probe.

Production of gluconic and ketogluconic acids from glucose

All strains of *G. hansenii* produced gluconic acid (Table 6). Furthermore, strains in groups I and IV produced 2- and 5-ketogluconic acids; the strain in group III produced 2-ketogluconic acid. Group II included two strains (IFO 14822=LMG 1517 and IFO 3261) that produced 2- and 5-ketogluconic acids and two strains (LMG 1688 and LMG 1689) that produced only 2-ketogluconic acid. Strains IFO 14816 and LMG 1536 were negative for gluconic acid and ketogluconic acid production, probably as a result of their very poor growth during cultivation in the prescribed medium. No strains produced 2,5-diketogluconic acid from glucose.

Discussion

The strains used in this study were the same as those employed in the establishment of *A. hansenii* (= *G. hansenii*) by Gosselé et al. (1983). These strains were shown to be heterogeneous on the basis of quinones, DNA base composition, DNA relatedness, production of gluconic and ketogluconic acids from glucose, and phenotypic characteristics. The strain IFO 3296 that originated from *A. pasteurianus* subsp. *pasteurianus* should be transferred to the genus *Acetobacter* because of the presence of Q-9. Within the family *Acetobacteraceae*, only the genus *Acetobacter* has Q-9 as the major ubiquinone (Yamada, 1983; Yamada and Kondo, 1984; Yamada et al., 1968, 1997).

Strains of *G. hansenii* were separated into four DNA homology groups (I, II, III, and IV), all of which were distinct from other species of the genus *Gluconacetobacter* investigated in this study, and from *Acetobacter*

Table 4. DNA base composition and levels of DNA relatedness in *Gluconacetobacter hansenii* strains.

Strain No.	mol% G+C	Levels of DNA relatedness ^a (%) with strain										Homology group
		IFO 14820 ^T	LMG 1527 ^T	IFO 14817	IFO 14822	LMG 1517	LMG 1688	LMG 1689	IFO 3261	LMG 1582	IFO 14815	
IFO 14820 ^T	59.0	100	95	105	13	24	11	13	29	14	27	I
LMG 1527 ^T	59.2	113	100	116	20	20	20	22	25	33	51	I
IFO 14817	58.9	88	83	100	11	11	10	9	24	10	37	I
IFO 14822	60.8	8	11	14	100	78	67	78	57	17	34	II
LMG 1517	60.7	8	11	16	106	100	75	81	76	16	38	II
LMG 1688	60.9	23	24	31	71	94	100	106	66	40	55	II
LMG 1689	60.4	14	14	18	112	93	92	100	57	40	51	II
IFO 3261	62.2	26	24	9	88	75	76	61	100	43	20	II
LMG 1582	60.8	23	23	29	36	37	32	32	49	100	29	III
IFO 14815	61.7	44	39	45	48	59	46	49	55	30	100	IV

Note: DNA relatedness was not determined in strains IFO 14816 and LMG 1536 because of the difficulty in DNA extraction posed by the extreme production of cellulose.
^a Homology values are averages of at least three determinations and were subjected to Q-test statistics in rejecting data.
^T Type strain.

Table 5. Levels of DNA relatedness of *Gluconacetobacter hansenii* strains with other species of acetic acid bacteria.

Species and strain No.	Homology group	Levels of DNA relatedness ^a (%) with strain						
		<i>G. xylinus</i> LMG 1515 ^T	<i>G. liquefaciens</i> AJ 12950 ^T	<i>G. diazotrophicus</i> NCIMB 12985	<i>A. aceti</i> IFO 14818 ^T	<i>A. pasteurianus</i> LMG 1262 ^T	<i>A. pasteurianus</i> IFO 13752	<i>Gl. oxydans</i> ^b IFO 14819 ^T
<i>G. hansenii</i>								
IFO 14820 ^T	I	3	5	ND ^c	9	19	2	25
LMG 1527 ^T	I	5	13	16	8	20	ND	15
IFO 14817	I	2	7	8	6	25	9	20
IFO 14822	II	1	6	10	6	11	ND	17
LMG 1517	II	2	6	ND	8	43	ND	41
LMG 1688	II	4	15	19	12	18	ND	27
LMG 1689	II	3	19	6	7	15	ND	23
IFO 3261	II	10	6	ND	20	23	ND	ND
LMG 1582	III	3	17	14	9	23	ND	25
IFO 14815	IV	4	10	16	10	28	ND	32

Note: Probes gave 100% levels of DNA relatedness with themselves.
^a Homology values are averages of at least three determinations and were subjected to Q-test statistics in rejecting data.
^b *Gluconobacter oxydans*.
^c ND: not determined.
^T Type strain.

Table 6. Oxidation products from glucose by *Gluconacetobacter hansenii* strains.

Strain No.	Homology group	Oxidation products ^a			
		Gln	2-kgln	5-kgln	2,5-dikgln
IFO 14820 ^T	I	+	+	+	—
LMG 1527 ^T	I	+	+	+	—
IFO 14817	I	+	+	+	—
IFO 14822	II	+	+	+	—
LMG 1517	II	+	+	+	—
LMG 1688	II	+	+	—	—
LMG 1689	II	+	+	—	—
IFO 3261	II	+	+	+	—
LMG 1582	III	+	+	—	—
IFO 14815	IV	+	+	+	—

^a Gln: gluconate; 2-kgln: 2-ketogluconic acid; 5-kgln: 5-ketogluconic acid; and 2,5-dikgln: 2,5-diketogluconic acid.

^T Type strain.

aceti, *A. pasteurianus*, and *Gluconobacter oxydans*. The type strain of *G. hansenii* was included in homology group I; thus only this group should be regarded as *G. hansenii*. The other homology groups (II, III, and IV) are probably new species based on the recommendation of the ad hoc committee of the International Committee for Systematic Bacteriology (Wayne et al., 1987).

The high levels of DNA relatedness within each homology group, especially in groups I and II, clearly indicate taxonomic homogeneity within a group. For instance, strains included in group I are those formerly designated as *A. aceti* subsp. *orleanensis*, i.e., IFO 14820^T (=LMG 1527^T and type strain of *G. hansenii*) and IFO 14817. These strains were celluloseless mutants of NCIB 8745, which is a variant of *A. aceti* subsp. *xylinum* NCIB 8246 (=IFO 14816). These strains displayed great similarities in their protein patterns (Gosselé et al., 1983). It is unfortunate, however, that because of the difficulty in DNA extraction, DNA relatedness with IFO 14816 cannot be determined to ascertain the genetic similarity of this strain with IFO 14820^T and IFO 14817. In the same context, all strains of group II are those formerly considered as *A. aceti* subsp. *xylinum* strains.

We confirmed the previous findings of Gosselé et al. (1983) that strains of *A. hansenii* (= *G. hansenii*) were unable to grow on ethanol as a sole carbon source. This is significant because this physiological feature can be used as a distinguishing characteristic between strains of *G. hansenii* and strains of *A. aceti* that can grow on ethanol. However, the addition of 0.05% yeast extract to the Hoyer-Frateur medium resulted in the growth of *G. hansenii* strains. The production of acid from ethanol was also recognized by the use of the same medium supplemented with

Bromocresol Purple as an indicator of acid production (data not shown). It is clear that *G. hansenii* strains have the ability to oxidize ethanol to acetic acid, despite their inability to use ethanol as a sole carbon source. This indicates that the strains require essential factors for growth. The oxidation of ethanol (or any other process, for that matter) cannot proceed without prior growth of a microorganism.

The strains of *G. hansenii* arose from strains formerly classified as *A. aceti* subsp. *orleanensis*, *A. aceti* subsp. *xylinum*, and *A. pasteurianus* subsp. *pasteurianus*. The strains of *A. aceti* subsp. *orleanensis* had not been well characterized and were scattered in two species, namely, *A. hansenii* (= *G. hansenii*) and *A. pasteurianus* (Gosselé et al., 1983). Further study is needed to clarify the taxonomic position of *A. aceti* subsp. *orleanensis*. *A. aceti* subsp. *xylinum* had been well characterized as *Gluconacetobacter xylinus* (Navarro and Komagata, 1999) and was distinct from *G. hansenii* on the basis of DNA relatedness.

Since this study included all the strains employed for the establishment of *Acetobacter hansenii* (= *G. hansenii*) by Gosselé et al. (1983), our genotypic and phenotypic data indicate that the species *G. hansenii* should be restricted to DNA homology group I. Further studies will be conducted to identify the other homology groups that are possibly new species. This is asserted because all strains did not hybridize with the type strains of known species of *Gluconacetobacter*, *Acetobacter*, and *Gluconobacter*.

Recently, *A. pomorum*, *A. oboediens* (Sokollek et al., 1998), and *A. intermedius* (Boesch et al., 1998) were described. However, Yamada et al. (2000) reported that *A. pomorum* was included in the sublineage of the genus *Acetobacter*, whereas, *A. oboediens* and *A. intermedius* were included in the sublineage of the genus *Gluconacetobacter*. Therefore *A. oboediens* and *A. intermedius* are regarded as *Gluconacetobacter* species. DNA-DNA hybridization between strains of *G. hansenii* and the type strains of the above three species, including *G. europaeus*, was not done in this study because of the extreme difficulty in culturing strains of these species. Nevertheless, previous reports have shown the taxonomic distinction of these four species with *G. hansenii*. Sievers et al. (1992), Sokollek et al. (1998), and Boesch et al. (1998) reported the genotypic distinction of the type strain of *G. hansenii* with the other four species on the basis of data of DNA-DNA relatedness and 16S rRNA sequence analysis. Moreover, strains of *G. hansenii* do not require acetic acid for growth, have Q-10 do not use ethanol as a sole source of carbon, do not grow in the presence of 30% glucose, and do not require high relative humidity for growth, in contrast with strains of *G. europaeus* (Sievers et al., 1992), *A. po-*

morum, *A. oboediens* (Sokollek et al., 1998), and *A. intermedius* (Boesch et al., 1998).

It is evident from the above discussion that the value of DNA relatedness between strains comprising a new species should be determined in the designation of the species. Thus current bacterial species without data of DNA relatedness should be reexamined for the stability of the bacterial names.

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