Gluconobacter oxydans subsp. *sphaericus*, New Subspecies Isolated From Grapes

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A new subspecies, *Gluconobacter oxydans* subsp. *sphaericus*, is described. This subspecies differs from the other subspecies of *G. oxydans*, the only species recognized in the genus *Gluconobacter*, both in its morphology and in its vitamin requirement for growth: the cells of the new subspecies are spherical at any age of culture and in all environments tested, and the organism requires nicotinic acid for growth; it differs from all other gluconobacters in that it does not require pantothenic acid. The type strain is IFO 12467.

During a survey by Ameyama in 1966 of the acetic acid bacteria, a hitherto undescribed organism was isolated from fresh grapes. The cells of this organism were spherical at any age of culture and in all media tested. With but a few exceptions, the organism had the physiological and biochemical characteristics of organisms belonging to the species *Gluconobacter oxydans*. The present paper describes the results of a taxonomic study of this new organism.

MATERIALS AND METHODS

Bacterial strains. The strains studied and their sources are listed in Table 1. The strains other than the strain of the newly isolated organism were obtained from the Institute of Fermentation, Osaka (IFO). In the tables of this paper, these strains are listed by the names under which they were received.

Media. Stock cultures were maintained on potato-glycerol (PG) slants. This medium was prepared as follows: 200 g of freshly sliced potato was boiled in 1 liter of tap water and autoclaved for 10 min at 2 kg/cm²; dried yeast extract (Oriental Yeast Co.) (10 g), polypeptone (10 g), glycerol (20 g), and glucose (5 g) were added to the potato extract.

Media used for determining cultural characteristics were PG medium, 0.8% nutrient broth (Difco) containing 1% glycerol, and a synthetic medium (S) containing 5 g of carbohydrate as the sole carbon source, 5 g of vitamin-free Casamino Acids (Difco), 0.4 mg of nicotinic acid, 0.5 g of KH₂PO₄, 0.5 g of K₂HPO₄, 0.2 g of MgSO₄·7H₂O, and 10 mg each of NaCl, FeSO₄, and MnSO₄·7H₂O in 1 liter. All media were sterilized for 30 min at 2 kg/cm².

Vitamin requirement determinations. The effect on growth of nicotinic acid, pantothenic acid, *p*-aminobenzoic acid, and thiamine (0.4 mg/liter) was examined upon their addition to glucose-S medium. Inocula were taken from cultures grown to the stationary phase (48 h) in PG medium, which had been prepared by dispensing 2.5-ml quantities into 8-ml centrifuge tubes and autoclaving. The inocula were washed twice with sterilized water by centrifugation and suspended in the same water. One drop of the

suspension served as inoculum for a test tube of the culture medium, which was dispensed in 6-ml quantities into test tubes (18 by 150 mm, for use in colorimetry). The cultures were incubated with reciprocal shaking at 30 °C for 3 days. Growth was measured by optical density in a Hitachi model EBO-B photometer with a 660-nm filter.

Synchronous culture. Cells grown for 2 days on PG slants and washed twice with sterilized water were inoculated into nicotinic acid-free glycerol-S medium. After incubation for 3 h, sterilized nicotinic acid solution (up to 0.4 mg/liter) was added to the cultures.

Oxidation of carbohydrates and amino acids. Oxidation of carbohydrates and amino acids was carried out in a conventional Warburg apparatus at 30 °C. Each Warburg vessel contained 1.0 ml of cell suspension, 1.5 ml of 0.067 M phosphate buffer (pH 6.0), 0.3 ml of substrate solution, and 0.2 ml of 15% KOH (in the center well). The substrate solution contained 30 μmol of carbohydrate or 5 μmol of amino acid. Substrates examined were glucose, sodium succinate, sodium acetate, ethanol, DL-alanine, sodium L-glutamate, and L-serine.

Detection of 2,5-diketogluconate and γ-pyrone compounds. Cells were inoculated in S medium containing 2% sodium gluconate. The cultures were incubated with reciprocal shaking at 30 °C for 2 days. The cells were removed by centrifugation, and the supernatant was subjected to paper chromatography. 2,5-Diketogluconate on paper chromatograms was detected with 2,3,5-triphenyl-tetrazolium-chloride and *p*-anisidine as the developing agents (7). With these developing agents, 2- and 5-ketogluconates were also detected. γ-Pyrone compounds were detected with ferric chloride as the developing agent (1).

Microscope observations. Bacteria were stained with the Ziehl carbol-fuchsin and the Hucker Gram-staining solutions and observed with a light microscope. The presence of flagella was determined by the Leifson staining method (9) and also by electron microscopy. For electron microscopy, cells grown on PG slant cultures for 18 to 24 h at 30 °C were subjected to observation with an electron microscope. After having been shadowed with germanium, the

TABLE 1. *Strains studied and their sources*

Strain	Source
Acetobacters	
<i>Acetobacter aceti</i> subsp. <i>aceti</i> (<i>A. aceti</i>)	IFO 3281
<i>A. aceti</i> subsp. <i>liquefa-</i> <i>ciens</i> (<i>G. liquefaciens</i>)	IFO 12388
<i>A. pasteurianus</i> subsp. <i>ascendens</i> (<i>A. ascendens</i>)	IFO 3188
Not described in the 8th ed. of <i>Bergey's</i> <i>Manual</i>	
<i>A. rancens</i>	IFO 3298
<i>A. acetigenus</i>	IFO 3280
<i>A. aurantium</i>	IFO 3246
Gluconobacters	
<i>Gluconobacter oxydans</i> subsp. <i>oxydans</i> (<i>G. oxydans</i>)	IFO 3189, 3287
<i>G. oxydans</i> subsp. <i>industrius</i> (<i>G. industrius</i>)	IFO 3260
(<i>G. capsulatus</i>)	IFO 3462
<i>G. oxydans</i> subsp. <i>suboxydans</i> (<i>G. gluconicus</i>)	IFO 3171, 3286
(<i>G. dioxycetonicus</i>)	IFO 3271, 3272, 3273
(<i>G. nonoxygluconicus</i>)	IFO 3276
(<i>G. cerinus</i>)	IFO 3262, 3263, 3264
(<i>G. cerinus</i>) var. (<i>ammoniacus</i>)	IFO 3267
(<i>G. albidus</i>)	IFO 3251, 3252
(<i>G. suboxydans</i>)	IFO 3289, 3291
(<i>G. suboxydans</i>) var α	IFO 3257
(<i>G. oxydans</i>) subsp. <i>melanogenes</i> (<i>G. melanogenus</i>)	IFO 3292, 3293
(<i>G. rubiginosus</i>)	IFO 3243, 3244
(<i>G. oxydans</i>) subsp. <i>sphaericus</i> subsp. n.	Fresh grapes

cells were viewed in a Hitachi model HS-6 electron microscope.

RESULTS

The gram-negative, diffusible-pigment-producing strain isolated from fresh grapes was similar to *Gluconobacter oxydans* subsp. *melanogenes* and *Acetobacter aceti* subsp. *liquefaciens*. The physiological and biochemical characteristics of the grape strain were compared with those of other acetic acid bacteria (*Gluconobacter* and *Acetobacter*).

The results of aerobic growth and other physiological tests are presented in Tables 2 and 3. Cultural characteristics are given in the de-

TABLE 2. *Aerobic growth of Gluconobacter oxydans subsp. sphaericus with various substrates*

Substrate	Growth	Substrate	Growth
Ethanol	+	Fructose	+
Butanol	—	Sorbose	+
Glycerol	+	Mannitol	+
DL-Arabinose	—	Sorbitol	+
Xylose	+	Sucrose	+
Rhamnose	—	Maltose	—
Glucose	+	Starch	—
Galactose	+	Dextrin	—
Mannose	+	Inulin	—

scription of this new strain presented below.

The results given in the tables agree with those obtained with hitherto described organisms belonging to the genus *Gluconobacter*.

Vitamin requirement tests for growth. Ameyama and Kondo (3) tested the vitamin requirement for growth of the acetic acid bacteria and reported that gluconobacters require one or more vitamins but acetobacters do not require any. Gluconobacters always require pantothenic acid (some strains require nicotinic acid, *p*-aminobenzoic acid, and/or thiamine in addition), but they do not require vitamins B₂, B₆, B₁₂, folic acid, biotin, or inositol. Therefore, in this study, nicotinic acid, pantothenic acid, *p*-aminobenzoic acid, and thiamine were examined for their effect, if any, on the growth of the newly isolated strain.

The experimental results are shown in Tables 4 and 5. The newly isolated strain required only nicotinic acid and thus differed from all other strains belonging to the genus *Gluconobacter*. Growth of the newly isolated strain was satisfied by the addition of at least 0.1 mg of nicotinic acid per liter to the medium (Fig. 1).

Oxidation of carbohydrates and amino acids. As described previously (2, 4, 8, 10), gluconobacters do not oxidize pyruvate, acetate, or members of the Krebs cycle. Furthermore, they do not oxidize amino acids (5, 6).

The results of testing for the oxidation of carbohydrates and amino acids are presented in Table 6. From these results, the newly isolated strain was clearly identified as belonging to *Gluconobacter*.

Detection of 2,5-diketogluconate and γ -pyrone compounds. To compare the newly isolated strain with *Gluconobacter oxydans* subsp., *melanogenes* (*G. melanogenus* IFO 3293), *Acetobacter aceti* subsp. *liquefaciens* (*G. liquefaciens* IFO 12388), and *A. aurantium* IFO 3246 (4, 8), cultures of the strains were incubated in sodium gluconate-S medium and the excreted products were detected by paper chromatography. All four of the pigment-producing strains

TABLE 3. *Physiological characteristics of Gluconobacter oxydans subsp. sphaericus*

Characteristic	Result	Medium
Catalase	+	PG
Amylase	—	PG with 2% starch
Reaction in milk	—	Litmus milk with 40 mg of nicotinate per liter
Nitrite production	±	Glycerol-S
Indole production	—	Glycerol-S
Hydrogen sulfide production	—	Glycerol-S
Growth with 12% NaCl	—	PG
Voges-Proskauer	±	Glucose-S
Methyl red	+	Glucose-S
Pigment formation	Red brown	Glucose-S
	—	Glycerol-S
Oxygen demand	Strictly aerobic	PG
Motility	—	PG with 0.5% agar

TABLE 4. *Vitamin requirements for growth of Gluconobacter oxydans subsp. sphaericus*^a

Vitamins added to glucose-S medium				Optical density
NA	PA	PABA	B ₁	
x	x	x	x	0.354
x	x	x		0.323
x	x		x	0.350
x		x	x	0.360
x				0.355
	x	x	x	0.020
				0.027

^a Symbols and abbreviations: x, vitamin added; NA, nicotinic acid; PA, pantothenic acid; PABA, *p*-aminobenzoic acid; B₁, thiamine.

used in this experiment produced 2- and 5-ketogluconates, 2,5-diketogluconate, and one or more γ -pyrone compounds.

Morphological characteristics. As described in *Bergey's Manual of Determinative Bacteriology* (7th ed.), individual cells of acetic acid bacteria are generally ellipsoidal to rod shaped, and the spherical cells may be considered to be a kind of involution form. Cells of the newly isolated strain, however, are always spherical, with a diameter of 1.3 to 1.4 μ m, in any growth phase and in any medium (Fig. 2). One of the cells shown in Fig. 2 appears to be undergoing fission. Observation of old cultures in the light microscope revealed that dividing cells sometimes appeared rod shaped. The spherical shape of the cells was confirmed by

TABLE 5. *Vitamin requirements for growth of gluconobacters*^a

Strain	Vitamin required for growth			
	NA	PA	PABA	B ₁
<i>G. oxydans</i> IFO 3287	x	x		
<i>G. industrius</i> IFO 3260		x	x	
<i>G. capsulatus</i> IFO 3462	x	x		
<i>G. gluconicus</i> IFO 3286		x		
<i>G. dioxycetonicus</i> IFO 3272		x	x	
IFO 3273	x	x		
<i>G. nonoxygluconicus</i> IFO 3276		x	x	
<i>G. cerinus</i> IFO 3262		x	x	
IFO 3263, 3264		x		
<i>G. cerinus</i> var. <i>ammoniacus</i> IFO 3267	x	x	x	x
<i>G. albidus</i> IFO 3251		x	x	
IFO 3252	x	x		
<i>G. suboxydans</i> IFO 3289, 3291		x	x	
<i>G. melanogenus</i> IFO 3292	x	x		x
IFO 3293	x	x		
<i>G. rubiginosus</i> IFO 3243	x	x		
IFO 3244	x	x		x
<i>G. oxydans</i> subsp. <i>sphaericus</i> subsp. n.	x			

^a Symbols and abbreviations: x, vitamin required; NA, nicotinic acid; PA, pantothenic acid; PABA, *p*-aminobenzoic acid; B₁, thiamine.

successive observations on many kinds of media during the 9 years of study on this organism since it was isolated from fresh grapes in 1966.

Synchronous culture. As mentioned above, some older cells appeared to be rod shaped under a light microscope. Therefore, to confirm that the cell shape is spherical, growing cells were observed in synchronous culture inoculated from old PG slants. These cells, even under a light microscope, were all sphere shaped

FIG. 1. Relation between the growth of *Gluconobacter oxydans* subsp. *sphaericus*, expressed in units of optical density, and concentration of nicotinic acid in glucose-S medium.

immediately after fission, and the cell shape was always spherical in any stage of one generation (generation time: about 3 h) up to the next fission.

DISCUSSION

The newly isolated bacterium described here is very similar physiologically and biochemically to organisms belonging to the genus *Gluconobacter* (Tables 2, 3, and 6). It is placed in the genus *Gluconobacter* Asai as a new subspecies of *G. oxydans* because it is a strict aerobe, is catalase positive, is methyl red positive, grows well between pH 3.5 and 6.0, and oxidizes ethanol to acetic acid and glucose to keto-gluconates via gluconate, but not succinate, acetate, or alanine. This organism differs from other subspecies of the species *G. oxydans* heretofore described in its spherical cell shape and in its distinctive vitamin requirement for growth. Table 7 presents the characteristics which differentiate the five subspecies of *G. oxydans* from each other and from a pigment-producing acetobacter.

The new organism produces the same diffusible pigment and the same γ -pyrone compounds from glucose or gluconate as do *G. oxydans* subsp. *melanogenes*, *A. aceti* subsp. *liquefaciens*, and *A. aurantium*. As shown in Table 6, strains producing the same pigment occur in both *Acetobacter* and *Gluconobacter*. For this

TABLE 6. Oxidation of carbohydrates and amino acids by acetic acid bacteria

Strain	Oxidative activity (Q_{O_2}) for						
	Glucose	Succinate	Acetate	Ethanol	Alanine	Glu-ta-mate	Serine
Acetobacters							
<i>Acetobacter aceti</i> IFO 3281	100	250	110	300	.15	1	1
<i>Gluconobacter liquefaciens</i> ^a IFO 12388	108	110	100	120	5	1	4
<i>Acetobacter ascendens</i> IFO 3188	44	70	13	106	10	1	3
<i>A. rancens</i> IFO 3298	84	227	80	230	8	1	1
<i>A. kuetzingianus</i> IFO 3222	130	195	100	200	5	1	2
<i>A. acetigenum</i> IFO 3280	60	198	100	235	7	2	3
<i>A. aurantium</i> ^a IFO 3246	160	130	40	150	7	1	6
Gluconobacters							
<i>Gluconobacter oxydans</i> IFO 3189	188	0	0	220	0	0	0
<i>G. capsulatus</i> IFO 3462	150	0	0	210	0	0	0
<i>G. gluconicus</i> IFO 3171	240	0	0	288	0	0	0
<i>G. dioxyacetonicus</i> IFO 3271	202	0	0	242	0	0	0
<i>G. cerinus</i> IFO 3264	206	0	0	289	0	0	0
<i>G. albidus</i> IFO 3251	212	0	0	276	0	0	0
<i>G. suboxydans</i> var. α IFO 3257	200	0	0	104	0	0	0
<i>G. melanogenus</i> ^a IFO 3292	222	0	0	200	0	0	0
<i>G. rubiginosus</i> ^a IFO 3243	200	0	0	180	0	0	0
<i>G. oxydans</i> subsp. <i>sphaericus</i> ^a	200	0	0	220	0	0	0

^a Produce diffusible pigment in glucose-S medium.

FIG. 2. Electron micrograph of *Gluconobacter oxydans* subsp. *sphaericus* IFO 12467, showing spherical shape. Bar indicates 2 μ m.

TABLE 7. Characteristics useful for differentiating between the five subspecies of *Gluconobacter oxydans* and *Acetobacter aceti* subsp. *liquefaciens*

Differentiating characteristics	<i>G. oxydans</i> subsp:					<i>A. aceti</i> subsp. <i>liquefaciens</i>
	<i>oxydans</i>	<i>industrius</i>	sub- <i>oxydans</i>	<i>melanogenes</i>	<i>sphaericus</i>	
Cell shape	Rod	Rod	Rod	Rod	Sphere	Rod
γ -Pyrone production from glucose	—	—	—	+	+	+
Vitamin requirement for growth:						
Pantothenic acid	+	+	+	+	—	—
Nicotinic acid	$\pm$	$\pm$	$\pm$	$\pm$	+	—
Polysaccharide formation from sucrose or dextrin	—	+	—	—	—	—

reason, the new organism should not be placed in *G. oxydans* subsp. *melanogenes*.

***Gluconobacter oxydans* subsp. *sphaericus* subsp. n.**

sphaericus. Gr. adj. *sphaericus* spherical.

Spheres, 1.3 to 1.4 μ m in diameter, occurring singly. Gram negative. Nonmotile.

Catalase positive.

Gelatin stab: Uniform line of puncture and no liquefaction.

Agar colonies: Opalescent, circular, pulvinate, smooth, entire.

Agar slant: Opalescent, filiform, smooth, raised.

Broth: Ring surface growth, slight clouding, and scanty compact sediment.

Grows very well between pH 3.5 and 6.0.

Nicotinic acid, but not pantothenic acid, required for growth.

Strict aerobe.

Oxidizes glycerol, xylose, glucose, galactose, mannose, fructose, sorbose, sorbitol, mannitol, and sucrose.

Oxidizes ethanol to acetic acid.

Does not oxidize acetic acid, succinic acid, DL-alanine, L-glutamic acid, or L-serine.

Pigment, γ -pyrone compounds, and 2,5-diketoglucuronate are produced from glucose or glucuronate.

The type strain has been deposited in the Institute of Fermentation, Osaka (IFO) under the number 12467. This strain was isolated in 1966 from fresh grapes. Because the subspecies description given above is based on a single strain, the type strain, it serves also as the description of the type strain.

REPRINT REQUESTS

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