INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, July 1997, p. 825–831 Vol. 47, No. 3
0020-7713/97/$04.00+0

Propionibacterium cyclohexanicum sp. nov., a New Acid-Tolerant ω-Cyclohexyl Fatty Acid-Containing Propionibacterium Isolated from Spoiled Orange Juice

KAORI KUSANO,[1]* HIDEKO YAMADA,[1] MOTOHIRO NIWA,[1] AND KAZUHIDE YAMASATO[2]

R&D Office, KIRIN Beverage Corporation, Samukawa-machi, Koza-gun, Kanagawa Prefecture 253-01,[1] and Culture Collection Center, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156,[2] Japan

A non-spore-forming, coryneform bacterium, strain TA-12^T, was isolated from spoiled off-flavor orange juice. Growth of this organism occurs at pH 3.2 to 7.5, and optimum growth occurs at pH values between 5.5 and 6.5. This organism produces lactic acid, propionic acid, and acetic acid from glucose. It is catalase negative. The cells are heat resistant and can withstand a temperature of 90°C for 10 min. The DNA G+C content is 66.8 mol%. This strain has an MK-9(H_4) respiratory quinone system and contains *meso*-diaminopimelic acid in its cell wall, and ω-cyclohexyl undecanoic acid is the major cellular fatty acid. The results of a phylogenetic analysis of the 16S rRNA gene of this organism indicated that its highest level of homology is its level of homology with the representative of the classical propionibacteria, *Propionibacterium freudenreichii* (97.1%). Strain TA-12^T is phenotypically similar to *P. freudenreichii*, but it produces a large amount of lactic acid and has a distinct fatty acid composition, acid tolerance, and heat resistance, which differentiate it from *P. freudenreichii* and other propionic acid-producing bacteria. On the basis of these findings we propose the name *Propionibacterium cyclohexanicum* sp. nov. for this organism. The type strain is TA-12 (= IAM 14535 = NRIC 0247).

Microbial contamination and growth in consumer products is one of the major problems in the food industry. It has been generally recognized that the major microbial contaminants in the fruit juice industry are yeasts, molds, and lactic acid bacteria, as these organisms prefer or can tolerate the low pH values of fruit juices. The growth of contaminant bacteria other than lactic acid bacteria in packaged juices was not reported until about 10 years ago. In 1984, spore-forming bacteria which can germinate and grow in apple juice were found by Cerny et al. (2), and similar spore formers were isolated from fruit juices in 1994 (26). The organism isolated by Cerny et al. was named *Bacillus acidoterrestris* by Deinhard et al. (8) and was reclassified as *Alicyclobacillus acidoterrestris* by Wisotzkey et al. (35) in 1992. The genus *Alicyclobacillus* is acidophilic and either thermophilic or thermotolerant, and characteristically the members of this genus contain ω-alicylic fatty acid.

In 1993, we isolated a coryneform bacterium, strain TA-12^T, from spoiled orange juice. Like members of the genus *Alicyclobacillus*, this organism is acid tolerant and ω-cyclohexyl fatty acid is its major cellular fatty acid, but it does not form spores.

In this paper we present a taxonomic characterization of this bacterium and propose the name *Propionibacterium cyclohexanicum* for it.

MATERIALS AND METHODS

Bacterial strains. Strain TA-12^T was isolated from spoiled off-flavor orange juice by using pH 7.0 Standard Method Agar ("Nissui" medium; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) incubated at 35°C. This medium contained (per liter) 5 g of peptone, 2.5 g of yeast extract, 1 g of glucose, and 15 g of agar. *Propionibacterium freudenreichii* ATCC 6207^T was used as a reference strain. Strain TA-12^T has been deposited in the IAM Culture Collection at the Institute of Molecular and Cellular Biosciences (formerly Institute of Applied Microbiology), University of Tokyo, as strain IAM 14535^T and in the Culture Collection Center, Tokyo University of Agriculture, as strain NRIC 0247^T.

Media and cultivation. Strain TA-12^T was cultivated in PYG medium containing (per liter) 5 g of peptone (Difco Laboratories, Detroit, Mich.), 2.5 g of yeast extract (Difco Laboratories), and 5 g of glucose. The pH was adjusted to 6.5 by adding sulfuric acid prior to autoclaving. For solid medium, 1.5% agar was added. Strain TA-12^T and *Propionibacterium freudenreichii* ATCC 6207^T were grown in stationary cultures at 35 and 30°C, respectively. For chemotaxonomic studies strains were cultured in medium containing (per liter) 5 g of peptone, 2.5 g of yeast extract, and 1 g of glucose (pH 6.5). The optimal temperature and optimal pH were determined by measuring the optical density at 580 nm.

Morphological features. Gram staining was carried out by the modified Hucker method (11). Spore staining was carried out by the modified method of Schaeffer and Fulton (25).

Physiological and biochemical features. Anaerobic growth was tested by using an Anaeropouch (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). Oxidase production was tested by using a cytochrome oxidase test strip (Nissui Pharmaceutical Co., Ltd.). Other tests were conducted by using the methods described by Cowan and Steel (5). Assimilation of carbon compounds was determined by observing acid production with an API 50CH kit (BioMerieux S.A., Marcy l'Etoile, France) by the method of Deinhard et al. (8), with some modifications. To minimize ambiguous observations, low concentrations of peptone were used in the basal medium (0.01% peptone for strain TA-12^T and 0.05% peptone for *Propionibacterium freudenreichii* ATCC 6207^T). The indicator used was bromocresol purple. When the results were ambiguous, we retested without the indicator by measuring the decrease in pH after 1 week of incubation. When the pH was less than 3.8, a sugar test was considered positive. Assimilation of acetic, lactic, malic, citric, and succinic acids was determined by culturing cells in test tubes containing PYG medium without glucose and measuring the amount of growth.

Heat resistance. The heat resistance of cells was determined by the screw-cap tube technique described by Kooiman (16). A 0.1-ml portion of a stationary-phase PYG broth culture, whose cell concentration was 10^{10} cells/ml, was injected into 10 ml of preheated fresh medium in a screw-cap test tube. Test tubes were preheated at various temperatures and were maintained at these temperatures for various periods of time. Cell viability was determined by examining the growth exhibited by the heated tube cultures.

Fermentation products. The fermentation products were analyzed by high-performance liquid chromatography (HPLC). Strain TA-12^T was cultured in Trypticase-yeast extract-glucose broth (pH 7.0) (6) at 35°C for 4 days. After the cells were removed by filtration through a 0.2-μm-pore-size membrane filter, the broth was used in the analysis. The HPLC system used was a 9000 series (Japan Spectroscopic Co., Ltd., Hachioji, Japan) equipped with a Shodex KC-811 column (Showa Denko K. K., Tokyo, Japan). The samples were eluted at 50°C with 3 mM perchloric acid (1.0 ml/min) and reaction buffer (1.5 ml/min) containing 0.2 mM bromothymol blue, 15 mM Na_2HPO_4, and 2 mM NaOH. Organic acids were detected by A_{445}.

Cell wall composition. Peptidoglycan was prepared as follows. About 1 g of wet cells was resuspended in 10 ml of 0.05 M phosphate buffer (pH 7.2) and sonicated with a Handy Sonic instrument (model UR-20D; Tomy Seiko Co., Ltd., Tokyo, Japan) for 20 min at power level 10. Then the sample was centri-

* Corresponding author. Fax: 81-467-74-6025. E-mail: k-kusano @kirin.co.jp..beverage.co.jp.

fuged at 5,000 × *g* for 10 min. The supernatant was boiled with 4% sodium dodecyl sulfate for 40 min and ultracentrifuged at 100,000 × *g* for 30 min. The precipitated proteins were digested with a solution containing 200 μg of pronase E (Sigma Chemical Co., St. Louis, Mo.) per ml, 3 mg of trypsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) per ml, and 3 mg of pepsin (Sigma Chemical Co.) per ml, and the lipids were removed by a series of ether-ethanol (1:1), chloroform-methanol (2:1), chloroform, and acetone extractions. The DNA and RNA were digested with solutions containing 40 μg of DNase (Boehringer Mannheim GmbH, Mannheim, Germany) per ml and 40 μg of RNase (Boehringer Mannheim GmbH) per ml, respectively. The sample was finally purified by trichloroacetic acid treatment (12). The amino acid composition of the purified peptidoglycan was determined by the method described by Cohen and Michaud (4). The diaminopimelic acid isomers were analyzed by the method described by Staneck and Roberts (28). The glycolyl residue was determined by the method of Uchida and Aida (34).

Whole-cell sugar pattern. Cell sugars were analyzed by the method described by Staneck and Roberts (28).

Respiratory quinone system. Menaquinones were extracted and analyzed by the method described by Tamaoka et al. (31).

Fatty acid analysis. Cell lipids were prepared from about 1 g of wet cells by the Bligh-Dyer method (1). The cells were broken with a Waring blender containing glass beads (80 to 100 mesh), and the lipids were extracted with a chloroform-methanol mixture (2:1). The extracted lipids were hydrolyzed at 60°C for 5 min with 0.5 N NaOH dissolved in methanol and then methylated at 60°C for 5 min with methanol containing 7% boron trifluoride (19). Gas chromatography-mass spectrometry was performed with a model 5971A mass spectrometer (Hewlett-Packard GmbH, Waldboron, Germany) and a model 5890 gas chromatograph (Hewlett-Packard GmbH) equipped with a type DB-23 capillary column (J&B Scientific, Inc., Folsom, Calif.). The column temperature was increased from 130 to 210°C at a rate of 3°C/min.

Extraction and purification of DNA. About 1 g of wet cells was resuspended in 15 ml of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0), and 5 mg of lysozyme (Wako Pure Chemical Industries, Ltd.), 2,000 U of achromopeptidase (Wako Pure Chemical Industries, Ltd.), and 30 μl of 5 M NaCl (final concentration, 0.01 M) were added. This mixture was incubated at 37°C for 1 h and then at 70°C for 10 min. Total lysis was achieved by adding 375 μl of 20% sodium dodecyl sulfate. Proteins were digested by overnight incubation at 55°C with 1.5 mg of proteinase K (Wako Pure Chemical Industries, Ltd.). The residual proteins and polysaccharides were removed by hexadecyltrimetylammonium bromide treatment (18). Further purification was carried out by repeated incubation at 55°C with proteinase K, deproteinization with phenol and chloroform-isoamyl alcohol (24:1), and precipitation with isopropyl alcohol.

Measurement of the G+C content of the DNA. Purified DNAs were hydrolyzed into nucleosides and analyzed by HPLC by the method of Tamaoka and Komagata (30), with some modifications. The heat-denatured DNA solution was incubated at 50°C overnight with nuclease P1 (Yamasa Shoyu Co., Ltd., Choshi, Japan). The HPLC system used was a 9000 series (Japan Spectroscopic Co., Ltd.) equipped with a CrestPak C18T-5 column (Japan Spectroscopic Co., Ltd.), and the nucleosides were eluted with 10 mM KH_2PO_4 (pH 3.5).

Determination of the 16S rRNA gene sequence and phylogenetic analysis. The 16S rRNA gene was amplified in vitro by PCR (23) by using a 16S rRNA gene-specific primer pair consisting of 5′-TTGGATCCAGAGTTTGATCCTG GCTCAG-OH-3′ (positions 8 to 27; *Escherichia coli* numbering system [9]), which contains a *Bam*HI site, and 5′-TTAAGCTTAAGGAGGTGATCCAGC CGCA-OH-3′ (positions 1521 to 1540; *E. coli* numbering system), which contains a *Hin*dIII site. Each cycle consisted of 1 min of denaturation at 94°C, 2 min of annealing at 55°C, and 3 min of extension at 72°C. A total of 30 cycles were followed by rapid cooling to 4°C. The amplified fragments were digested with *Bam*HI and *Hin*dIII. The digests were checked for length by 1% agarose gel electrophoresis and were purified by extraction with a Geneclean II kit (Bio 101, Inc., La Jolla, Calif.). The purified DNA fragments were ligated with plasmid vector pUC119 which had been double digested with *Bam*HI and *Hin*dIII by using a DNA ligation kit (Takara Shuzo Co., Ltd., Kyoto, Japan). Subcloning was carried out by digestion with restriction enzymes *Eco*RI, *Sal*I, and *Sph*I. The cloned DNAs were purified by polyethylene glycol precipitation (33). The purified plasmids were sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer Co., Norwalk, Conn.) and were analyzed with a model ABI 373A DNA sequencer (Perkin-Elmer Co.). The primary sequences of the TA-12^T 16S rRNA gene were generated by aligning and combining partial sequences by using DNASIS software (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). The DNA sequences which ranged from positions 261 to 1455 (*E. coli* numbering system) were aligned on the basis of the secondary structure of the 16S rRNA. Positions at which the bases were uncertain and at which gaps were generated by the comparative alignment were omitted from the analysis. A total of 806 nucleotides were used. A phylogenetic tree was constructed by using Clustal W software (32).

Nucleotide sequence accession numbers. The sequence of *Propionibacterium cyclohexanicum* TA-12^T (accession no. D82046) was aligned with previously published sequences obtained from the EMBL, GenBank, and DDBJ databases under the following accession numbers: *Aeromicrobium erythreus*, M37200; *Arthrobacter globiformis* DSM 20124^T, M23411; *Luteococcus japonicus* IFO 12422^T, D21245; *Micrococcus luteus* ATCC 381, M38242; *Nocardioides albus* DSM 43109^T, X53211; *Propionibacterium acidipropionici* DSM 20272, X53221; *Propionibacterium acnes* DSM 1897^T, X53218; *Propionibacterium freudenreichii* DSM 20271^T, X53217; *Propionibacterium jensenii* DSM 20535^T, X53219; *Propionibacterium propionicus* DSM 43307^T, X53216; *Propionibacterium thoenii* DSM 20276^T, X53220; *Terrabacter tumescens* NCIB 8914^T, X53215; and *Bacillus subtilis*, X00007. The sequence of the 16S ribosomal DNA gene of *Propioniferax innocua* NCTC 11082^T was obtained from reference 20.

RESULTS

Morphological features. Strain TA-12^T was a gram-positive, nonmotile, pleomorphic, rod-shaped bacterium whose cells were 1.5 to 3.0 μm long and 1.1 to 1.6 μm wide. The cells were club shaped and bent, and some swellings were observed. Spores were not observed. The colonies on the surfaces of PYG agar plates after 3 days of anaerobic growth were circular, white to creamy, translucent, and 0.2 to 0.5 mm in diameter.

Physiological and biochemical features. Lactic acid, propionic acid, and acetic acid were formed as a result of fermentation at a molar ratio of 5:4:2, respectively. The physiological, biochemical, and chemotaxonomic characteristics of strain TA-12^T and *Propionibacterium* species are shown in Table 1. Some data for *Propionibacterium freudenreichii* ATCC 6207^T obtained in this study are included in Table 1. *Propionibacterium freudenreichii* ATCC 6207^T was catalase positive, but strain TA-12^T was catalase negative. Strain TA-12^T was aerotolerant, forming colonies on the surface of agar plate medium incubated aerobically. When cells were suspended and grown in semisolid medium, growth occurred evenly throughout the medium except for the surface layer, where it was less vigorous. Both strain TA-12^T and strain ATCC 6207^T grew at 20 to 40°C, and the optimum growth temperatures were 35°C for strain TA-12^T and 30 to 32°C for *Propionibacterium freudenreichii*. Strain TA-12^T grew at pH 3.2 to 7.5, and the optimum pH was 5.5 to 6.5. Strain ATCC 6207^T grew at pH 4.5 to 8.5, and optimum growth occurred at pH 6.5 to 7.0. Strain TA-12^T was resistant to heating at 90°C for 10 min. The comparative carbon compound assimilation profiles of *Propionibacterium* species are shown in Table 2. Strain TA-12^T could assimilate 23 of the 54 carbon compounds tested, including glycerol, L-xylose, galactose, D-glucose, D-fructose, D-mannose, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, saccharose, trehalose, melezitose, D-turanose, D-fucose, acetic acid, lactic acid, malic acid, citric acid, and succinic acid, whereas *Propionibacterium freudenreichii* ATCC 6207^T assimilated 14 carbon compounds, including glycerol, erythritol, ribose, adonitol, galactose, D-glucose, D-fructose, D-mannose, inositol, acetic acid, lactic acid, malic acid, citric acid, and succinic acid.

Chemotaxonomic features. The major menaquinone of strain TA-12^T was MK-9(H_4) and the G+C content was 66.8 mol%. The major cellular fatty acid was ω-cyclohexyl undecanoic acid, which accounted for 52.7% of the total fatty acids. The straight-chain and anteiso-branched fatty acids included 16.8% *n*-C_{15}, 6.4% anteiso-C_{15}, 2.8% *n*-C_{16}, and 5.3% *n*-C_{17}, and the other fatty acids were not determined. The cell wall peptidoglycan contained *meso*-diaminopimelic acid, glutamic acid, and alanine at a molar ratio of 1:1:2. The whole-cell sugars were galactose, mannose, glucose, ribose, and rhamnose. An unidentified sugar spot was observed; the R_f value of this sugar was 0.38, which is almost the R_f value of fucose on a chromatogram.

Phylogenetic analysis. Table 3 shows the 16S rRNA gene similarity values for strain TA-12^T and allied gram-positive bacteria with high G+C contents. The level of similarity between strain TA-12^T and *Propionibacterium freudenreichii* DSM 20271^T was 97.1%. The levels of homology with other

TABLE 1. Biochemical and chemotaxonomic characteristics of strain TA-12^T and *Propionibacterium* spp.

Characteristic	Strain TA-12^T	*Propionibacterium freudenreichii*[a]	*Propionibacterium jensenii*[a]	*Propionibacterium thoeniii*[a]	*Propionibacterium acidipropionici*[a]	*Propionibacterium acnes*[a]	*Propionibacterium avidum*[a]	*Propionibacterium granulosum*[a]	*Propionibacterium lymphophilum*[a]	*Propionibacterium propionicus*[b]
Catalase	−[c]	+	d+	+	d+	d+	+	+	d+	−
Relationship to oxygen	Aerotolerant	Anaerobic to aerotolerant	Anaerobic to aerotolerant	Less strictly anaerobic	Anaerobic to aerotolerant	Anaerobic to aerotolerant	Anaerobic or microaerophilic	Anaerobic or microaerophilic	Anaerobic	Facultatively anaerobic
Hydrolysis of gelatin	−	−	−	−	−	+	+	d−	d+	d
Hydrolysis of starch	−	−	−	d+	−	−	−	−	d−	d
Hydrolysis of esculin	+	+	+	+	+	−	+	−	−	−
Hydrolysis of urea	+	−[d]	ND[e]	ND	ND	ND	ND	ND	ND	−
Reduction of nitrate to nitrite	−	d	−	−	+	d+	−	−	d	+
Formation of indole	−	−	−	−	−	d+	−	−	−	−
pH range for growth	3.2-7.5	4.5-8.5[d]	ND	ND	ND	ND	ND	ND	ND	ND
Optimum temp for growth (°C)	35	30-32	30-32	30-32	30-32	36-37	36-37	36-37	36-37	35-37
Fermentation products	Lactic acid, propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Acetic acid, propionic acid, lactic acid, succinic acid
Major respiratory quinone	MK-9(H_4)	MK-9(H_4)[f]	MK-9(H_4)[f]	MK-9(H_4)[f]	MK-9(H_4)[f]	MK-9(H_4)[f]	MK-9(H_4)[f]	MK-9(H_4)[f]	MK-9(H_4)[f]	MK-9(H_4)
G+C content (mol%)	66.8	64-67	65-68	66-67	66-68	57-60	62-63	61-63	53-54	63-65
Isomer of A_2pm in cell wall[g]	*meso*-A_2pm	*meso*-A_2pm	LL-A_2pm	LL-A_2pm	LL-A_2pm	LL-(*meso*)-A_2pm	LL-(*meso*)-A_2pm	LL-A_2pm		LL-A_2pm
Major fatty acid	ω-Cyclohexane	Branched	ND	ND	Branched	Branched	Branched	Branched	Branched	Straight
Sugars in polysaccharide[h]	Glucose, galactose, mannose, rhamnose, ribose	Galactose, mannose, rhamnose	Glucose, galactose, mannose	Glucose, galactose	Galactose, glucose, mannose	Glucose, mannose, (galactose)	Glucose, mannose, (galactose)	Galactose, mannose	Galactose, glucose, mannose	Glucose, galactose, mannose
Amino acids in cell wall[g]	Alanine, glutamic acid, *meso*-A_2pm	Alanine, glutamic acid, *meso*-A_2pm	Alanine, glutamic acid, glycine, LL-A_2pm	Alanine, glutamic acid, glycine, LL-A_2pm	Alanine, glutamic acid, glycine, LL-A_2pm	Alanine, glutamic acid, (glycine), LL-(*meso*)-A_2pm	Alanine, glutamic acid, (glycine), LL-(*meso*)-A_2pm	Alanine, glutamic acid, glycine, LL-A_2pm	Alanine, glutamic acid, lysine	Glycine, glutamic acid, alanine, LL-A_2pm

[a] Data from reference 7.
[b] Data from references 3 and 24.
[c] −, 90% or more of the strains are negative; +, 90% or more of the strains are positive; d, 11 to 89% of the strains are positive; d+, 40 to 90% of the strains are positive; d−, 10 to 40% of the strains are positive.
[d] Data for strain ATCC 6207^T determined in this study.
[e] ND, no data available.
[f] Data from reference 14.
[g] A_2pm, diaminopimelic acid.
[h] Sugars in parentheses are absent in some strains.

TABLE 2. Acid formation from various carbon sources by strain TA-12[T] and *Propionibacterium* spp.

Carbon source	Acid formation by:									
	Strain TA-12[T]	*Propionibacterium freudenreichii*[a]	*Propionibacterium jensenii*[a]	*Propionibacterium thoenii*[a]	*Propionibacterium acidipropionici*[a]	*Propionibacterium acnes*[a]	*Propionibacterium avidum*[a]	*Propionibacterium granulosum*[a]	*Propionibacterium lymphophilum*[a]	*Propionibacterium propionicus*[b]
Glycerol	+[c]	+	+	+	+	d+	+	+	−	d
Erythritol	−	+	+	d+	+	d+	+	−	+	d
D-Arabinose	−	+, −[d]	−	−	+	−	d+	−	−	−
Ribose	−	d+	+	+	+	d+	d+	d−	+	d
D-Xylose	−	−	d+	d+	d+	−	d−	−	−	−
L-Xylose	+	−[d]	ND[e]	ND	ND	ND	ND	ND	ND	ND
Adonitol	−	d+	d+	d+	+	d+	d+	−	+	d
Galactose	+	+	+	+	+	d+	+	d−	−	d
D-Glucose	+	+	+	+	+	d+	+	+	+	+
D-Fructose	+	+	+	+	+	d+	+	+	+	+
D-Mannose	+	+	+	+	+	d+	+	+	−	d
L-Sorbose	−	−	−	−	d+	−	−	−	−	−
Rhamnose	−	−	−	−	+	−	−	−	−	−
Dulcitol	−	−	−	−	−	−	−	−	−	−
Inositol	−	d+	d+	d+	+	d−	d+	−	d+	d
Mannitol	−	−	+	−	+	d−	d−	d+	−	+
Sorbitol	−	−	−	d+	+	d+	−	−	−	d
Amygdalin	+	−	d+	d+	−	−	−	d−	−	d
Arubtin	+	−[d]	ND	ND	ND	ND	ND	ND	ND	ND
Esculin	+	−	−	+	d+	−	−	−	−	ND
Salicin	+	−	+	d+	+	−	d+	−	−	d
Cellobiose	+	−	d−	−	+	−	−	−	−	−
Maltose	+	−	d+	d+	+	−	+	d+	+	+
Lactose	+	d−	d+	d−	+	−	d+	−	−	d
Melibiose	±	d−	+	d+	d+	−	d+	d−	−	d
Saccharose	+	−	+	d+	+	−	+	+	d−	+
Trehalose	+	−	+	+	+	−	+	d+	−	d
Inulin	−	−	−	−	−	−	−	−	−	−
Melezitose	+	−	d+	d+	+	−	d+	d−	−	−
D-Raffinose	−	−	+	d+	d−	−	d+	d+	−	+
Starch	−	−	−	+	+	−	−	−	d+	d
Glycogen	−	−	−	d+	−	−	−	−	−	−
D-Turanose	+	−[d]	ND	ND	ND	ND	ND	ND	ND	ND
D-Fucose	+	−[d]	ND	ND	ND	ND	ND	ND	ND	ND

[a] Data from reference 7.
[b] Data from references 3 and 24.
[c] +, 90% or more of the strains are positive; −, 90% or more of the strains are negative; d, 11 to 89% of the strains are positive; d+, 40 to 90% of the strains are positive; d−, 10 to 40% of the strains are positive.
[d] Data for strain ATCC 6207[T] determined in this study.
[e] ND, no data available.

TABLE 3. 16S rRNA gene sequence similarity values for strain TA-12^T and related species

Species[a]	% Similarity														
	Propionibacterium freudenreichii	*Propionibacterium propionicus*	*Propionibacterium thoenii*	*Propionibacterium acidipropionici*	*Propionibacterium acnes*	*Propionibacterium jensenii*	*Propioniferax innocua*	*Luteococcus japonicus*	*Aeromicrobium erythreum*	*Terrabacter tumescens*	*Nocardioides albus*	*Streptomyces griseus*	*Arthrobacter globiformis*	*Micrococcus luteus*	*Bacillus subtilis*
Propionibacterium cyclohexanicum	97.1	95.7	95.0	94.9	94.9	94.9	94.3	93.4	92.4	91.9	91.8	91.3	90.9	90.6	81.5
Propionibacterium freudenreichii		95.8	95.4	95.5	95.3	95.3	94.2	92.7	92.9	91.2	91.8	90.9	91.2	90.9	82.0
Propionibacterium propionicus			95.7	95.8	95.5	95.5	94.8	93.9	93.3	92.7	93.2	91.8	91.7	91.1	82.5
Propionibacterium thoenii				98.3	96.0	98.6	93.3	92.5	93.3	92.4	91.8	91.3	91.8	91.8	82.5
Propionibacterium acidipropionici					96.3	98.0	93.0	91.3	92.9	91.6	91.6	90.7	91.2	91.2	83.0
Propionibacterium acnes						96.0	91.8	91.8	91.1	89.9	90.6	89.3	89.4	89.4	81.4
Propionibacterium jensenii							93.2	91.9	92.8	91.7	91.3	90.8	90.9	91.1	82.2
Propioniferax innocua								93.8	94.4	92.2	94.2	92.1	91.2	91.2	83.1
Luteococcus japonicus									92.4	91.8	92.4	90.6	90.6	89.9	82.4
Aeromicrobium erythreum										93.3	95.3	92.3	92.8	92.4	83.1
Terrabacter tumescens											92.2	93.3	94.7	94.5	84.1
Nocardioides albus												91.2	91.6	91.2	82.2
Streptomyces griseus													92.3	92.3	84.1
Arthrobacter globiformis														96.9	83.9
Micrococcus luteus															84.2

[a] The designations of the strains used are indicated in the text.

propionibacteria were around 95%, and the levels of homology with other bacteria were lower. Figure 1 shows a phylogenetic tree based on the 16S rRNA gene sequences of strain TA-12^T, *Propionibacterium* species, and other related groups. Our strain was located closest to *Propionibacterium freudenreichii*.

DISCUSSION

Strain TA-12^T is a gram-positive, nonmotile, pleomorphic, rod-shaped bacterium. It produces propionic acid from glucose. MK-9(H_4) is its respiratory quinone, and alanine, glu-

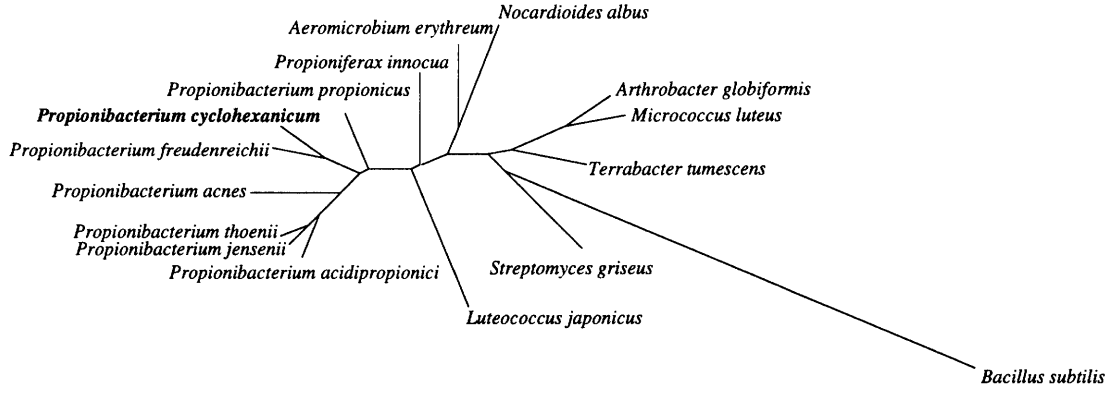

FIG. 1. Unrooted phylogenetic tree showing the positions of *Propionibacterium cyclohexanicum* TA-12^T and related species. The strains used are indicated in the text.

tamic acid, and *meso*-diamoinopimelic acid are components of the cell wall. The whole-cell sugars are galactose, mannose, glucose, ribose, and rhamnose. The G+C content of the DNA is 66.8 mol%. These morphological, biochemical, and chemotaxonomic features of strain TA-12^T are consistent with the characteristics of the genus *Propionibacterium*. Phylogenetically, strain TA-12^T is closely related to *Propionibacterium freudenreichii* DSM 20271, the type strain of the type species of the genus *Propionibacterium*. On the basis of its features and its phylogenetic location close to *Propionibacterium freudenreichii* DSM 20271^T, strain TA-12^T belongs to the genus *Propionibacterium*. Strain TA-12^T differs from previously described *Propionibacterium* species and other propionic acid-producing species by containing ω-cyclohexyl fatty acid. ω-Cyclohexyl fatty acid is a major cellular fatty acid of strain TA-12^T, and this fatty acid composition is unlike any previously reported cellular fatty acid composition of gram-positive, high-G+C-content bacteria except the *Curtobacterium pusillum* cellular fatty acid composition (28). Unlike strain TA-12^T, *C. pusillum* contains MK-9 and D-ornithine (15). Strain TA-12^T produces lactic acid, which distinguishes it from all other species except *Propionibacterium propionicus*. These and other phenotypic features listed in Tables 1 and 2 clearly distinguish strain TA-12^T from other propionibacteria. Thus, strain TA-12^T is distinct and represents a new species of the genus *Propionibacterium*.

Strain TA-12^T exhibited the highest level of 16S rRNA similarity (97.1%) with *Propionibacterium freudenreichii*; this value is such a low one that this bacterium does not hybridize with any bacteria at the species level (27). Strain TA-12^T is distinct both genetically and phenotypically.

The observed heat resistance of strain TA-12^T (it is able to survive at 90°C for 10 min) is very high. Malik et al. (17) reported that *Propionibacterium freudenreichii* was the most heat resistant of the propionibacteria, but this organism was reported to be able to survive at 62.8°C for 30 min. In our test, *Propionibacterium freudenreichii* could survive at 80°C for 10 min but could not survive at 90°C for 10 min. Fruit juices are generally pasteurized at 90 to 95°C for 30 s to 1 min prior to packaging, and it is possible that heat-resistant bacteria such as strain TA-12^T can survive this treatment. Conversely, isolation of strain TA-12^T from pasteurized and packaged orange juice substantiates its heat resistance. Despite the extraordinarily high resistance of strain TA-12^T to heat, no spores similar to the endospores of members of the *Bacillaceae* and no specialized structures were observed by optical microscopy of spore-stained cells or electron microscopy of thin sections of cells. This bacterium is a very unusual propionibacterium as it is able to grow at a low pH and is able to withstand heat treatment at 90°C for 10 min.

Alicylic fatty acid is a rare cellular fatty acid. ω-Cyclohexyl fatty acid has been found in *Bacillus acidocaldarius* (22) and *Bacillus acidoterrestris* (10), and ω-cycloheptyl fatty acid has been obtained from *Bacillus cycloheptanicus* (21). These three species are all acidothermophilic and were classified as members of the genus *Alicyclobacillus* in 1992 (35). In 1981, Suzuki et al. reported that *C. pusillum*, a gram-positive, high-G+C-content bacterium, contains ω-cyclohexyl fatty acid (29). This bacterium was isolated from oil brine (13) and is mesophilic and neutrophilic. Strain TA-12^T represents a third group of organisms which contain an alicylic fatty acid, and it is acidotolerant but not thermophilic. This shows that the presence of alicylic fatty acids in bacteria is not directly related to the apparent physiological properties of the organisms and is not related to what genus they belong to.

Description of *Propionibacterium cyclohexanicum* sp. nov. *Propionibacterium cyclohexanicum* (cy.clo.hex.a'ni.cum. Gr. n. *kyklos*, circle; Gr. n. *hexa*, six; M. L. masc. adj. *cyclohexanicum*, referring to ω-cyclohexyl fatty acid, the characteristic cellular fatty acid of the organism). Cells are gram-positive, aerotolerant, nonmotile, non-acid-fast, non-spore-forming, pleomorphic rods. The cells are 1.5 to 3.0 μm long and 1.1 to 1.6 μm wide. Some cells are club shaped or bent. Colonies grown anaerobically on the surface of peptone-yeast extract-glucose agar for 3 days are circular, white to creamy, translucent, and 0.2 to 0.5 mm in diameter. The catalase, oxidase, and Voges-Proskauer tests are negative, and the methyl red test is positive. Urea and esculin are hydrolyzed. Gelatin and starch are not hydrolyzed. Nitrate is not reduced to nitrite, and indole is not produced. Glycerol, L-xylose, galactose, D-glucose, D-fructose, D-mannose, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, saccharose, trehalose, melezitose, D-turanose, D-fucose, acetic acid, lactic acid, malic acid, citric acid, and succinic acid are assimilated. Erythritol, D-arabinose, ribose, adonitol, β-methylxyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methylmannoside, α-methylglucoside, *N*-acetylglucosamine, inulin, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, L-fucose, L-arabitol, gluconate, 2-ketogluconate, and 5-ketogluconate are not assimilated. Lactic acid and propionic acid are the major end products of the glucose fermentation, and acetic acid is also detected. Grows at pH 3.2 to 7.5 (optimum pH, 5.5 to 6.5) and at 20 to 40°C (optimum temperature, 35°C). The cells are heat resistant and survive at 90°C for 10 min. The major menaquinone is MK-9(H_4). ω-Cyclohexyl undecanoic acid is the main cellular fatty acid. The isomer of diaminopimelic acid in the cell wall is *meso*-diaminopimelic acid. The *N*-glycolyl residue is not present. The cell wall amino acids are alanine, glutamic acid, and *meso*-diaminopimelic acid. The whole-cell sugars are galactose, glucose, mannose, ribose, and rhamnose. The G+C content of the DNA is 66.8 mol%. The bacterium was isolated from pasteurized orange juice.

The type strain is TA-12 (= IAM 14535 = NRIC 0247).

ACKNOWLEDGMENTS

We thank Akihiro Iwamatsu and Katsuya Okawa, Central Laboratories for Key Technology, KIRIN Brewery Co., Ltd., for the analysis of amino acids. We thank Tomonori Suzuki, Science University of Tokyo, for helpful suggestions regarding alignment of the sequence data and calculations of homology values.

REFERENCES

1. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
2. **Cerny, G., W. Hennlich, and K. Poralla.** 1984. Spoilage of fruit juice by bacilli: isolation and characterization of the spoiling microorganism. Z. Lebensm. Unters. Forsch. **179**:224–227.
3. **Charfreitag, O., M. D. Collins, and E. Stackebrandt.** 1988. Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int. J. Syst. Bacteriol. **38**:354–357.
4. **Cohen, S. A., and D. P. Michaud.** 1993. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal. Biochem. **211**:279–287.
5. **Cowan, S. T., and K. J. Steel.** 1965. Manual for the identification of medical bacteria. Cambridge University Press, London, United Kingdom.
6. **Cummins, C. S., and J. L. Johnson.** 1981. The genus *Propionibacterium*, p. 1894–1902. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The procaryotes, vol. 1. Springer-Verlag, New York, N.Y.
7. **Cummins, C. S., and J. L. Johnson.** 1986. Genus *Propionibacterium*, p. 1346–1353. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, Md.
8. **Deinhard, G., P. Blanz, K. Poralla, and E. Altan.** 1987. *Bacillus acidoterrestris* sp. nov., a new thermotolerant acidophile isolated from different soils. Syst. Appl. Microbiol. **10**:47–53.
9. **Ehresmann, C., P. Stiegler, P. Fellner, and J. P. Ebel.** 1972. The determination of the primary structure of the 16S ribosomal RNA of *Escherichia coli*. Biochimie **54**:901–967.

10. **Hippchen, B., A. Röll, and K. Poralla.** 1981. Occurrence in soil of thermo-acidophilic bacilli possessing ω-cyclohexane fatty acids and hopanoids. Arch. Microbiol. **129:**53–55.
11. **Hucker, G. J., and H. J. Conn.** 1923. Methods of Gram staining. Tech. Bull. N. Y. State Agric. Exp. Stn. **93:**3–37.
12. **Hughes, R. C.** 1965. The isolation of structural components present in the cell wall of *Bacillus licheniformis* N.C.T.C. 6346. Biochem. J. **96:**700–709.
13. **Iizuka, H., and K. Komagata.** 1965. Determination *of Brevibacterium*, *Arthrobacter*, *Micrococcus*, *Sarcina*, *Alcaligenes*, and *Achromobacter* isolated from oil-brines in Japan. J. Gen. Appl. Microbiol. **11:**1–14.
14. **Jones, D., and M. D. Collins.** 1986. Irregular, nonsporing gram-positive rods, p. 1261–1266. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, Md.
15. **Komagata, K., and K. Suzuki.** 1986. Genus *Curtobacterium*, p. 1313–1317. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, Md.
16. **Kooiman, W. J.** 1969. The screw-cap tube technique: a new and accurate technique for the determination of the wet heat resistance of bacterial spores, p. 87–92. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press, London, United Kingdom.
17. **Malik, A. C., G. W. Reinbold, and E. R. Vedamuthu.** 1968. An evaluation of the taxonomy of *Propionibacterium*. Can. J. Microbiol. **14:**1185–1191.
18. **Meade, H. M., S. R. Long, C. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
19. **Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka.** 1996. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. **38:**514–515.
20. **Pitcher, D. G., and M. D. Collins.** 1991. Phylogenetic analysis of some LL-diaminopimelic acid-containing coryneform bacteria from human skin: description of *Propionibacterium innocuum* sp. nov. FEMS Microbiol. Lett. **84:**295–300.
21. **Poralla, K., and W. A. König.** 1983. The occurrence of ω-cycloheptane fatty acids in a thermo-acidophilic bacillus. FEMS Microbiol. Lett. **16:**303–306.
22. **Rosa, M. D., A. Gambacorta, L. Minale, and J. D. Bu'Lock.** 1971. Cyclohexane fatty acids from a thermophilic bacterium. Chem. Comm. 1334–1334.
23. **Saiki, R. K., D. H. Gelfand, S. Stoffe, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
24. **Schaal, K. P.** 1986. The genus *Arachnia*, p. 1332–1342. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, Md.
25. **Schaeffer, A. B., and M. D. Fulton.** 1933. A simplified method of staining endospores. Science **77:**194.
26. **Splittstoesser, D. F., J. J. Churey, and C. Y. Lee.** 1994. Growth characteristics of aciduric sporeforming bacilli isolated from fruit juices. J. Food Prot. **57:**1080–1083.
27. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
28. **Staneck, J. L., and G. D. Roberts.** 1974. Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. **28:**226–231.
29. **Suzuki, K., K. Saito, A. Kawaguchi, S. Okuda, and K. Komagata.** 1981. Occurrence of ω-cyclohexyl fatty acids in *Curtobacterium pusillum* strains. J. Gen. Appl. Microbiol. **27:**261–266.
30. **Tamaoka, J., and K. Komagata.** 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. **25:**125–128.
31. **Tamaoka, J., Y. Katayama-Fujimura, and H. Kuraishi.** 1983. Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. J. Appl. Bacteriol. **54:**31–36.
32. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
33. **Treisman, R.** 1989. Purification of plasmid DNA, p.1.40–1.41. *In* J. Sambrook, E. F. Fritsch, and T. Maniatis (ed.), Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Uchida, K., and K. Aida.** 1984. An improved method for the glycolate test for simple identification of the acyl type of bacterial cell walls. J. Gen. Appl. Microbiol. **30:**131–134.
35. **Wisotzkey, J. D., P. Jurtshuk, Jr., G. E. Fox, G. Deinhard, and K. Poralla.** 1992. Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. Int. J. Syst. Bacteriol. **42:**263–269.