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Zymobacter palmae gen. nov., sp. nov., a new ethanol-fermenting peritrichous bacterium isolated from palm sap

Tomoyuki Okamoto[1], Hiroshi Taguchi[1], Katsumi Nakamura[1], Hiroshi Ikenaga[1], Hiroshi Kuraishi[2], Kazuhide Yamasato[3]

[1] Central Laboratories for Key Technology, Kirin Brewery Co. Ltd., Fukuura, Kanazawa-ku, Yokohama 236, Japan
[2] Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwai-cho, Fuchu 183, Japan
[3] Institute of Applied Microbiology, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan

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Abstract. *Zymobacter palmae* gen. nov., sp. nov. was proposed for a new ethanol-fermenting bacterium that was isolated from palm sap in Okinawa Prefecture, Japan. The bacterium is gram-negative, facultatively anaerobic, catalase-positive, oxidase-negative, nonspore-forming and peritrichously flagellated. It requires nicotinic acid for growth. It ferments hexoses, α-linked di- and tri-saccarides, and sugar alcohols (fructose, galactose, glucose, mannose, maltose, melibiose, saccharose, raffinose, mannitol and sorbitol). Fifteen percent of maltose in broth medium is effectively fermented, whereas glucose with a concentration higher than 10% delayed growth initiation and decreased growth rates. Maltose is fermented to produce ethanol and CO_2 with a trace amount of acids. Approximately 2 mol of ethanol are produced from 1 mol moiety of hexose of maltose. The organism possesses ubiquinone-9. The G + C content of the DNA is 55.8 ± 0.4 mol%. Major cellular fatty acids were palmitic and oleic acids and cyclopropanic acid of $C_{19:0}$. Characteristic hydroxylated acid was 3-hydroxy dodecanoic acid. The bacterium is distinct from other ethanol-fermenting bacteria belonging to the genera *Zymomonas* Kluyver and van Niel 1936 and *Saccharobacter* Yaping et al. 1990 with respect to chemotaxonomic and other phenotypic characters to warrant to compose a new genus and a new species. The type strain is strain T109 (= IAM 14233).

Key words: *Zymobacter palmae* – Maltose fermentation – Ethanol production – Peritrichous flagellation – *Zymomonas* – Ubiquinone-9 – Fuel alcohol – Clean energy

Ethanol-producing bacteria have attracted much attention for these years, because their growth rate is higher than that of *Saccharomyces* (Rogers et al. 1979) presently used for practical production of fuel alcohol in the United States or Brazil, and, with the advances in biotechnology (Sprenger 1993), has been expected to be used for the more economical production of ethanol.

Zymomonas (Kluyver and van Niel 1936) is a well-known bacterium to produce ethanol and has been used for making alcoholic beverages from plant sap in tropical area. Its growth rate and specific ethanol production are high. However, it was hard to be utilized for ethanol production from starch-containing materials or raffinose-containing beet, because its fermentable carbohydrates are limited to glucose, fructose and saccharose. For obtaining bacterial cultures that are able to ferment various sugars, bacteria were screened from various vegetative samples collected from subtropical and tropical area. A new ethanol-producing bacterium was isolated and taxonomically characterized.

Materials and methods

Bacterial strains and isolation

For isolation of bacteria, the samples of plant sap, fermented foods and alcoholic beverages were collected from various countries including Brazil, Indonesia, Japan and Thailand. Samples were diluted with sterile physiological saline and plated on the medium composed of 1% Bacto yeast extract (Difco Laboratories, Detroit, Mich., USA), 2% glucose, 0.2% KH_2PO_4 and 1.5% Bacto-agar (Difco), pH 6.0. To prevent fungal and yeast multiplication, the antibiotic Kabicidin (Wako Pure Chemical Industries, Osaka, Japan) was added to the medium at the concentration of 100 mg/l. For the selection of ethanol tolerant bacteria, 5% (v/v) ethanol was added to the medium. The ethanol productivity from maltose of isolates was screened by cultivating in broth medium composed of 1% Bacto yeast extract (Difco), 2% maltose and 0.2% KH_2PO_4, pH 6.0. The amount of ethanol produced was estimated with a Hitachi 655 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with an RI detector and Aminex HPX-87P column (Bio-Rad Laboratories, Richmond, Calif., USA). Four strains designated as T109, T124, T143 and T202 were obtained from palm sap in Okinawa Prefecture, Japan, and were subjected to taxonomic characterization.

Abbreviation: IAM–IAM Culture Collection, Institute of Applied Microbiology, The University of Tokyo

Correspondence to: T. Okamoto

Morphological, physiological and biochemical characters

Cell morphology, Gram reation and motility were determined by using cells grown in maltose yeast extract (MY) medium at 30 °C with standing. The medium consisted of 1% Bacto yeast extract (Difco), 2% maltose, 0.2% KH_2PO_4 and 0.5% NaCl, pH 6.0. Flagellation was observed by electron microscopy for cells grown on Brain heart infusion agar (Difco) supplemented with 1 mg/l nicotinic acid, 2% maltose and 10% liver infusion, pH 6.0, at 20 °C for 12 h. The liver infusion was prepared by gentle boiling of sliced cattle liver in water (100 g in 250 ml) for 30 min and filtrating through cloth. Cells were shadowed with platinum at an angle of 20° by Balzers BAF 301 (Balzers, Balzers, Liechtenstein). The flagellation was observed by using a model JEM 200CX transmission electron microscope (JEOL, Akishima, Japan) at 100 kV.

Conventional taxonomic features were determined according to the procedures described by Akagawa and Yamasato (1989), Cowan (1974) or Edwards and Ewing (1962). α-glucosidase was tested by the method of Eivazi and Tabatabai (1988). Test media that could not support the growth were supplemented with nicotinic acid and/or Bacto yeast extract (Difco) and maltose (see Results and discussion). Temperature and pH for growth were measured with MY broth medium. Temperature for growth was determined by using a temperature gradientor TN-12 (Advantec Toyo Kaisya, Tokyo, Japan). Growth factor determination and fermentation test were carried out in the basal liquid medium composed of 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.1% $(NH_4)_2SO_4$, pH 6.0. For determination of growth factor requirements, addition and omission tests of vitamins and vitamin-like active substances were carried out in the basal liquid medium containing 2% maltose. Substances tested were vitamin A, biotin, thiamine, riboflavin, Ca-pantothenate, pyridoxine, vitamin B_{12}, nicotinic acid, *p*-aminobenzoic acid, folic acid, ascorbic acid, vitamin K, protoheme, indoleacetic acid and β-indolelactic acid. Fermentation test of carbon compounds to produce ethanol was conducted in basal liquid medium containing 1 mg/l nicotinic acid. Carbon compounds were added by 2% as the final concentration. The experiments of ethanol fermentation process from maltose were carried out by cultivating in MY broth medium containing 15% maltose.

Chemotaxonomic characters

Quinone fractions were extracted with chloroform-methanol (2 : 1, v/v) from lyophilized cells harvested from MY broth culture. The extract was partially purified with a thin-layer chromatography on Kieselgel 60 F_{254} plate (Merck, Darmstadt, Germany) developed with petroleum benzine-diethyl ether (9 : 1, v/v). The quinone fractions were analyzed by high-performance liquid chromatography (HPLC) with a CrestPack C18S column (Japan Spectroscopic Co., Hachioji, Japan) at 275 nm. Methanol-isopropanol (2 : 1, v/v) was used as an eluent.

To determine cellular fatty acid composition, cells were harvested at the mid-log growth phase from cultures that were grown in MY broth at 30 °C with standing. Fatty acids were extracted from lyophilized cells with *n*-hexane after methylation with 5% HCl-methanol by using the procedure of Ohara et al. (1990). Methyl esters of polar and non-polar fatty acids were separated on Kieselgel 60 plate (Merck) using a hexane-diethyl ether (85 : 15, v/v) developing system. Fatty acid methyl esters were analyzed by the procedure described previously (Akagawa and Yamasato 1989) with minor modifications. The percentages of cellular fatty acid components were calculated on the basis of the total non-hydroxylated acids as described by Katayama-Fujimura et al. (1982), because of the different locations and functions of the hydroxylated and non-hydroxylated acids.

DNA was extracted and purified according to the procedure described by Saito and Miura (1963). After deproteinization with phenol, DNA solution was treated with chloroform-isoamyl alcohol (24 : 1, v/v) followed by precipitation of DNA with isopropanol (Marmur 1961). The G + C content of the DNA was determined by a HPLC method (Tamaoka and Komagata 1984). As the standard DNA, G + C content of DNA from *Escherichia coli* IAM 1264 (strain K-12) was defined 51.6 mol%.

16S rRNA analysis

The 16S rRNA gene of *Zymobacter palmae* T109 was amplified by PCR with two combinations of primers, 20F (AGTTTGATCCTG GCTCA)-920R (CGTCAATTCCTTTGAGT) and 519F (CA GCAGCCGCGGTAATAC)-1406R (ACGGGCGGTGTGTGC). The sequence was determined with the generated templates and Taq Dye Deoxy Terminator Cycle Sequencing Kit by using DNA sequencer, model 373 A (Applied Biosystems Division, Perkin-Elmer Corp., Foster City, Calif., USA). The sequencing reactions were carried out according to the protocol of the manufacture's directions. The primers for sequencing were as those described by Lane et al. (1985).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number D14555.

Results and discussion

All strains grew much better in standing than in shaking culture. Broth of MY medium was slightly turbid, became cloudy with flocculent cells at late log phase and then transparent with sedimentation of cells at stationary phase. No pellicle was formed. The colonies on MY agar plate were round, entire, smooth, opaque and milky white. Cells were gram-negative, facultatively anaerobic rods, 0.7 to 0.9 μm by 1.3 to 2.4 μm. They were motile with peritrichous flagella (Fig. 1) and nonsporeforming.

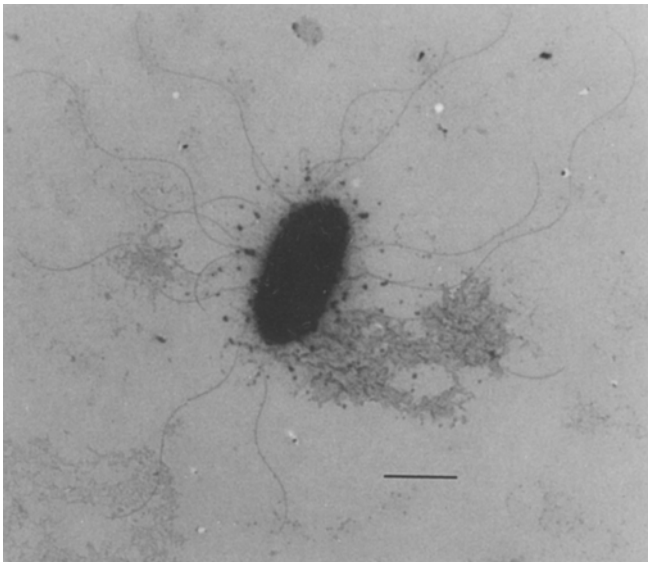

Fig. 1. Electron micrograph of peritrichous flagella of *Zymobacter palmae* T109. *Bar*, 1 μm

Table 1. Components of cellular fatty acids of *Zymobacter palmae*

Strains	Non-hydroxylated acid									Hydroxylated acid	
	12:0	14:0	15:0	16:0	16:1	17:0	18:0	(ω-9)-18:1	19 cyc	3-OH 10:0	3-OH 12:0
T109	5	4	tr	52	tr	tr	5	8	24	tr	12
T124	5	3	tr	46	tr	Tr	4	21	21	tr	8
T143	5	4	tr	51	tr	tr	6	8	26	tr	13
T202	6	4	tr	54	tr	tr	6	7	23	tr	13

The percentages of fatty acid compositions were calculated on the bases of total non-hydroxylated acids
Tr, between 0.5 and 1%; tr, less than 0.5%

Cells grew with a temperature of 21 to 39 °C and with a wide range of pH (initial) of 3 to 10. Optimum growth occurred at 30 °C and at pH 6.0. Catalase, methyl red, Voges-Proskauer and α-glucosidae (*p*-nitrophenyl-α-D-glucoside, PNPG) were positive. Production of oxidase, utilization of citrate, nitrate reduction, indole production, chromogenicity, gelatin liquefaction, hydrolysis of starch, phenylalanine deaminase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, β-galactosidase (*o*-nitrophenyl-β-D-glucoside, ONPG) were not detected. Only nicotinic acid was required for growth.

Respiratory quinone system was ubiquinone-9 and menaquinone could not be detected even in the lipid fraction extracted from 10 g of lyophilized cells. Whole cell fatty acid profiles were similar in all 4 strains (Table 1). They consisted of several saturated and unsaturated straight-chain fatty acids and 3-hydroxylated fatty acids. The main straight-chain acids were palmitic acid ($C_{16:0}$), oleic acid ($C_{(\omega-9)-18:1}$) and cyclopropanic acid of $C_{19:0}$ (C_{19cyc}). The amount of oleic acid from strain T124 was 2 or 3 times higher than that from other strains. Hydroxylated acid was characteristically 3-OH $C_{12:0}$.

The DNA base compositions of strains T109, T124, T143 and T202 were 56.2, 55.8, 55.4 and 56.0 mol% G + C, respectively.

Ethanol was produced from various carbon compounds; hexoses, α-linked di- and tri-saccharides, and sugar alcohols. When fermented, more than 0.5% of ethanol was produced in culture browth. As shown in Table 2, fructose, galactose, glucose, mannose, maltose, melibiose, saccharose, raffinose, mannitol and sorbitol were fermented, but arabinose, xylose, rhamnose, trehalose, cellobiose, lactose, dextrin and inulin were not.

Fermentation process of ethanol from maltose for the strain T109 is shown in Fig. 2. After the fermentation for 6 days in 100 ml broth containing 15.5 g of maltose,

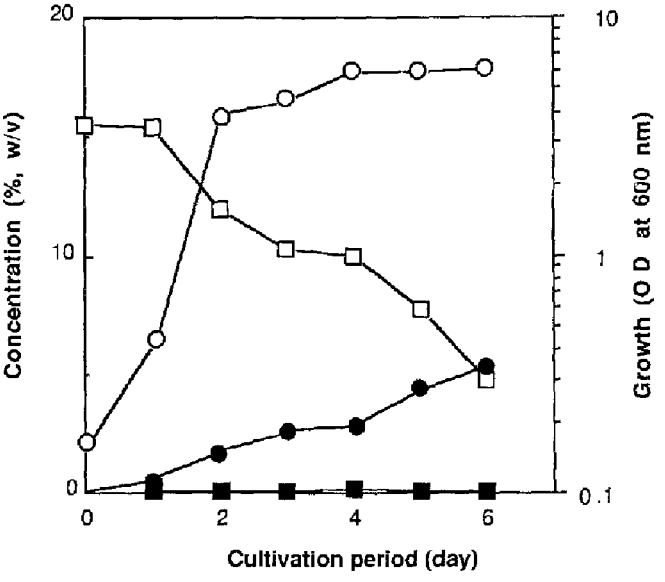

Fig. 2. Ethanol production by *Zymobacter palmae* T109. Concentration (%, w/v) in the browth: □ maltose, ■ glucose, ● ethanol. ○ Growth: OD_{600nm}

Table 2. Production of ethanol from carbohydrates and related compounds

Substrate	Strains			
	T109	T124	T143	T202
Galactose	+	−	+	−
Glucose	+	+	+	+
Mannose	−	+	+	−
Fructose	+	+	+	+
Maltose	+	+	+	+
Melibiose	+	+	−	+
Saccharose	+	+	+	+
Raffinose	+	+	+	+
Sorbitol	+	+	+	+
Mannitol	+	+	+	+

+, More than 0.5% ethanol was produced in culture broth. −, No ethanol was detected in culture broth

11.3 g of maltose were consumed and 5.8 g of ethanol were produced. As calculated from the yield of ethanol, approximate 2 mol of ethanol were produced from 1 mol of hexose moiety of maltose. The organism was considerably tolerant to ethanol; 5.8% of ethanol was produced for 6 days fermentation. The organism is distinct from the previously described ethanol-producing bacteria; the genera *Zymomonas* (Kluyver and van Niel 1936), and *Saccharobacter* (Yaping et al. 1990). All of them are gram-negative, facultatively anaerobic and nonsporeforming rods. With respect to flagellation, *Zymomonas* is polarly flagellated, and our organism and *Saccharobacter* are peritrichously flagellated. *Zymomonas* has ubiquinone-10, whereas our organism has ubiquinone-9. The DNA composition of our organism is 55.8 ± 0.4 mol% G + C that is different from 48.5 ± 0.5 mol% of *Zymomonas* and 63.5 ± 0.2 mol% of *Saccharobacter*. In physiological and biochemical characters, our organism differs from the both genera. *Zymomonas* requires biotin and Ca-pantothenate and ferments only glucose, fructose and saccharose. *Saccharobacter* differs in the ability to ferment pentoses, arginine dihydrolase, citrate utilization, phenylalanine deaminase, β-galactosidase and optimal temperature for growth (30–46 °C).

The sequence similarities of *Zymobacter palmae* T109 of the representatives of α-, β- and γ-groups of *Proteobacteria* including genera possessing ubiquinone-8, 9 and 10 were lower than 90%, as compared with sequence DATA from DDBJ.

The genera *Zymomonas* and *Saccharobacter* were described to be able to tolerate 40% (w/v) (Swings and De Ley 1983) and 35% (w/v) (Yaping et al. 1990) of glucose, respectively. As seen in Table 3, *Zymobacter palmae* T109 exhibited different attitudes toward glucose and maltose in terms of concentration in the medium. In the lower concentration, below 7.5% (w/v), specific growth rate was higher on glucose; it was nearly double on maltose. The rate on glucose decreased with increasing concentration and was very low at the higher than 15% (w/v). Whereas, the specific growth rate on maltose varied a little through 2.5 to 20% (w/v); it was 0.20 to 0.29 μ/h. The organism could initiate growth at 50% (w/v) of maltose, but not at 25% (w/v) of glucose. The different effect of osmotic pressure exerted by these sugars would be at least one of the reasons for the differences.

On the basis of taxonomic characteristics described above, the organism was concluded to compose a new genus and a new species. *Zymobacter palmae* was proposed for the organism.

The four strains studied, T109, T124, T143 and T202 were deposited in IAM with the accession numbers of IAM 14233, 14234, 14235 and 14236, respectively.

Description of Zymobacter gen. nov.

Zymobacter gen. nov.; Zy. mo. bac'ter Gr. n. *zyme* leaven, ferment; M. L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod; M. L. masc. n. *Zymobacter* the fermenting rod. Cells are gram-negative, facultatively anaerobic, nonsporeforming rods that are 0.7 to 0.9 μm by 1.3 to 2.4 μm.

Catalase-positive. Chemoorganotropic. Ferments 1 mol of glucose or hexose moiety of maltose to produce approximate 2 mol of ethanol and CO_2 with a trace amount of acids. Ferments hexoses, α-linked di- and tri-saccharides and sugar alcohols. Growth initiates at pH values of 3 to 10. The major cellular fatty acids are palmitic and oleic acids and cyclopropanic acid of $C_{19:0}$. Hydroxylated acid is characteristically 3-OH $C_{12:0}$. Quinone system is ubiquinone-9. The DNA base composition ranges from 55.4 to 56.2 mol% G + C (as determined by HPLC). The type species is *Zymobacter palmae*.

Description of Zymobacter palmae sp. nov.

Zymobacter palmae sp. nov; pal.'mae L. gen. n. *palmae* of palm. The description is the same as that for the genus. In addition, it has the characteristics described below. Colonies are round, entire, smooth, opaque and milky white. Methyl red, Voges-Proskauer and α-glucosidase are positive. The following tests are negative; indole production, utilization of citrate, nitrate reduction, chromogenicity, gelatin liquefaction, hydrolysis of starch, phenylalanine deaminase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and β-galactosidase. Fructose, galactose, glucose, mannose, maltose, melibiose, saccharose, raffinose, mannitol and sorbitol are fermented. Maltose is fermented to produce 5 to 6% (w/v) ethanol in culture broth. Temperature for growth ranges from 21 to 39 °C with an optimum of 30 °C. Optimum growth occurs at pH 6.0. Growth is better in standing than in shaking culture. The type strain is T109 (= IAM 14233).

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Table 3. Specific growth rates of *Zymobacter palmae* T109 as affected by the initial concentration of glucose and maltose

	Specific growth rate $\mu(h^{-1})$							
Sugar	2.5	5	7.5	10	12.5	15	17.5	20(%)
Glucose	0.47	0.43	0.38	0.26	0.19	0.08	0.07	0.05
Maltose	0.20	0.24	0.25	0.29	0.26	0.26	0.20	0.20

References

Akagawa M, Yamasato K (1989) Synonymy of *Alcaligenes aquamarinus*, *Alcaligenes faecalis* subsp. *homari*, and *Deleya aesta: Deleya aquamarina* comb. nov. as the type species of the genus *Deleya*. Int J Syst Bacteriol 39: 462–466

Cowan ST (1974) Cowman and Steel's manual for the identification of medical bacteria. Cambridge University Press. Cambridge

Edwards PR, Ewing WH (1962) Identification of *Enterobacteriaceae*. Burgess Publishing, Minneapolis, Minn

Eivazi F, Tabatabai MA (1988) Glucosidae and galactosidae in soil. Soil Biol Biochem 20: 601–606

Katayama-Fujimura Y, Tsuzaki N, Kuraishi H (1982) Ubiquinone, fatty acid and DNA base composition determination as a guide to the taxonomy of the genus *Thiobacillus*. J Gen Microbiol 128: 1599–1611

Kluyver AJ, Niel CB van (1936) Prospects for a natural system of classification of bacteria. Zentrabl Bakteriol Parasitenkd Infektionskr Hyg Abt 2 94: 369–403

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequence for phylogenetic analysis. Proc Natl Sci USA 82: 6955–6959

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol 3: 208–218

Ohara M, Katayama Y, Tsuzuki M, Nakamoto S, Kuraishi H (1990) *Paracoccus kocurii* sp. nov., a tetramethylammonium-assimilating bacterium. Int J Syst Bacteriol 40: 292–296

Rogers PL, Lee KJ, Tribe DE (1979) Kinetics of alcohol production by *Zymomonas mobilis* at high sugar concentrations. Biotechnol Lett 1: 165–170

Saito H, Miura K (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim Biophys Acta 72: 619–629

Sprenger GA (1993) Approaches to broaden the substrate and product range of the ethanologenic bacterium *Zymomonas mobilis* by genetic engineering. J Biotechnol 27: 225–237

Swings J, De Ley J (1983) Genus *Zymomonas* Kluyver and van Niel, 1936, 399. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Baltimore, pp 576–580

Tamaoka J, Komagata K (1984) Determination of DNA base composition by reverse-phase high-performance liquid chromatography. FEMS Microbiol Lett 25: 125–128

Yaping J, Xiaoyang L, Jiaqi Y (1990) *Saccharobacter fermentatus* gen. nov., sp. nov., a new ethanol-producing bacterium. Int J Syst Bacteriol 40: 412–414