Oenococcus kitaharae sp. nov., a non-acidophilic and non-malolactic-fermenting oenococcus isolated from a composting distilled shochu residue

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Six strains of lactic acid bacteria were isolated in Japan from a composting distilled shochu residue. The six isolates grew poorly on MRS agar and slowly in MRS broth. The 16S rRNA gene sequences did not show high levels of similarity to those of the recognized species of lactic acid bacteria, and formed a subcluster within the cluster comprising obligately heterofermentative lactic acid bacteria closely related to *Oenococcus oeni*. The levels of DNA–DNA relatedness revealed that the isolates belonged to the same taxon and were genetically separate from *O. oeni*. Furthermore, various phenotypic characteristics such as the optimum pH for growth, malolactic fermentation and resistance to 10 % ethanol revealed that the isolates are distinguishable from *O. oeni*. On the basis of their phylogenetic and phenotypic characteristics, the isolates represent a novel species, for which the name *Oenococcus kitaharae* sp. nov. is proposed. The type strain is NRIC 0645^T (=JCM 13282^T=DSM 17330^T).

During a study of lactic acid bacteria originating from fermented plant material, six strains of lactic acid bacteria were isolated from a composting distilled shochu residue produced in Japan. Shochu is produced in Japan from rice, sweet potato, barley and other starchy materials fermented using *Saccharomyces cerevisiae* and *Aspergillus niger*, and is produced mainly in the southern Kyushu region (Endo & Okada, 2005a). The composting represents an effective way of recycling the distilled shochu residue. The isolates grew poorly on MRS agar and slowly in MRS broth. A phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolates formed a subcluster within the cluster comprising the heterofermentative lactic acid bacteria (Collins *et al.*, 1991; Schleifer & Ludwig, 1995), and were most closely related to *Oenococcus oeni*. The levels of DNA–DNA relatedness demonstrated that there was a clear separation between the novel isolates and *O. oeni*. Furthermore, the isolates possessed various phenotypic characteristics that could be used to distinguish them from *O. oeni*. This work deals with the taxonomic analysis of these six isolates and shows that they represent a novel species of the genus *Oenococcus*.

The novel strains, NRIC 0645^T, NRIC 0646, NRIC 0647, NRIC 0648, NRIC 0649 and NRIC 0650, were isolated from a composting distilled shochu residue produced in Miyazaki

Prefecture in the southern Kyushu area of Japan: the isolation was performed on MRS (Oxoid) agar containing (l⁻¹) 10 mg cycloheximide, 10 mg sodium azide, 5.0 g calcium carbonate and 15 g agar at pH 6.8 and 30 °C. The strains formed very small colonies (less than 1 mm in diameter) on the MRS agar after incubation for 5 days. Colonies were picked and incubated in MRS broth (pH 6.8) at 30 °C. They grew slowly in the MRS broth, taking 4–5 days to reach the stationary phase. In MRS/tomato broth (MRS broth containing 10 % filtered tomato juice; pH 6.8) they took 4–5 days to reach the stationary phase, and in MRS/BHI broth [half-strength MRS broth containing half-strength brain heart infusion (Eikenkizai) broth; pH 6.8] they took 3–4 days. Therefore the isolates were maintained in MRS/BHI broth. *O. oeni* NRIC 0331^T was obtained from the NODAI Culture Collection Center at Tokyo University of Agriculture, and was maintained in MRS broth containing 10 % filtered tomato juice at pH 4.8 and 25 °C.

To determine the 16S rRNA gene sequences of the isolates, DNA extraction, amplification, purification and sequencing were carried out as described previously (Endo & Okada, 2005b). The closest recognized relatives of the isolates were determined by performing DataBase searches, and the sequences of closely related species were retrieved from the DDBJ database. Multiple alignments of the sequences were carried out with the program CLUSTAL_X, version 1.18 (Thompson *et al.*, 1997). Distance matrices for the aligned sequences were calculated by using the two-parameter

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NRIC 0645^T is AB221475.

method of Kimura (1980). The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). The robustness of individual branches was estimated by using bootstrapping with 1000 replicates (Felsenstein, 1985). To evaluate the topology of the phylogenetic tree, phylogenetic trees were also constructed by using the maximum-likelihood (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 1969) methods with PHYLIP version 3.65 (Felsenstein, 2005). Bootstrap values were determined by using the SEQBOOT program within the PHYLIP package. The 16S rRNA gene sequence (positions 8–1541) of strain NRIC 0645^T and partial sequences (positions 8–531) of strains NRIC 0646, NRIC 0647, NRIC 0648, NRIC 0649 and NRIC 0650 were determined. Position numbers were based on the *Escherichia coli* numbering system (GenBank accession no. V00348; Brosius *et al.*, 1981). The 16S rRNA gene sequence determined for strain NRIC 0645^T was used to search for sequence similarity with DataBase, and approximately 1450 bp of the 16S rRNA gene sequences of strain NRIC 0645^T and related species were used to construct a phylogenetic tree. The highest level of sequence similarity to strain NRIC 0645^T was found with *O. oeni* strains (96.0%). In particular, the similarity of a partial sequence of strain NRIC 0645^T [positions 8–531, containing the V1–V3 region (Van de Peer *et al.*, 2000)] with respect to that of *O. oeni* was less than 93%. This may be related to differences in the 16S rRNA secondary structures of these regions. The similarities for strain NRIC 0645^T with respect to *Leuconostoc* species and *Weissella* species were 82.8–85.5 and 82.8–84.7%, respectively. Partial sequences (positions 8–531) of the 16S rRNA gene were determined for strains NRIC 0646, NRIC 0647, NRIC 0648, NRIC 0649 and NRIC 0650; these partial sequences were identical to each another and to that of strain NRIC 0645^T (positions 8–531). Strain NRIC 0645^T formed a subcluster within the cluster comprising the obligately heterofermentative lactic acid bacteria (Collins *et al.*, 1991; Schleifer & Ludwig, 1995), and were most closely related to *O. oeni* on the basis of a neighbour-joining analysis (Fig. 1). Identical tree topologies were

obtained using the maximum-parsimony and maximum-likelihood methods (data not shown).

The levels of DNA–DNA relatedness between the isolates and *O. oeni* NRIC 0331^T were determined, and the DNA G + C contents were determined for the isolates. The extraction and isolation of bacterial DNAs were performed by using the method of Marmur (1961) as modified by Ezaki *et al.* (1983). DNA–DNA hybridization was carried out by using the microdilution well technique, with photobiotin for labelling of the DNA (Ezaki *et al.*, 1989). The G + C contents of the strains tested were determined by HPLC as described by Tamaoka & Komagata (1984). The isolates showed high levels of DNA–DNA relatedness (90–100%) to one another. Therefore, we concluded that the isolates belonged to the same taxon. In contrast, the isolates showed low levels of DNA–DNA relatedness (25–30%) to *O. oeni* NRIC 0331^T. The G + C contents of the isolates ranged from 41 to 43 mol%.

To differentiate the isolates, randomly amplified polymorphic DNA (RAPD) fingerprinting was performed. DNA extraction was carried out by using a method described previously (Endo & Okada, 2005b). RAPD PCR was performed according to the methods of Akopyanz *et al.* (1992) and Morotomi *et al.* (2002), using three primers (D, 5'-GAGGACAAAG; E, 5'-GGCGTCGGTT; and F, 5'-GGCCACGGAA). The PCR products were subjected to 1.5% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed under UV light. The fingerprinting indicated that the isolates were genetically different from one another (Fig. 2).

Morphological, physiological and biochemical characteristics were determined by using methods described previously (Endo & Okada, 2005b), but MRS/BHI broth was used as a basal medium. In addition, the decarboxylation of L-malate to L-lactate (malolactic fermentation) was determined by assessing the degradation of L-malate by using HPLC, as described by Iino *et al.* (2001). The detailed

Fig. 1. Neighbour-joining phylogenetic tree showing relationships between strain NRIC 0645^T and related taxa on the basis of 16S rRNA gene sequences. *Lactococcus lactis* ATCC 19435^T was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

Fig. 2. RAPD PCR fingerprinting of the six novel isolates. Primers D, E and F were used (see text for details). Lanes: M, size marker (500 bp DNA ladder, Takara Bio); 1, NRIC 0645^T; 2, NRIC 0646; 3, NRIC 0647; 4, NRIC 0648; 5, NRIC 0649; 6, NRIC 0650.

characteristics of the isolates are presented in the species description and Table 1. They formed small colonies (1 mm in diameter) on MRS/BHI agar after incubation for 7 days and formed very small colonies (less than 1 mm in diameter) on MRS agar after incubation for 7 days under aerobic conditions; they formed large colonies (2 mm in diameter) on both media under anaerobic conditions in anaerobic jars (GasPak System; BBL) after incubation for 5 days. The isolates produced D-lactic acid, carbon dioxide and ethanol or acetic acid from D-glucose. This is consistent with *O. oeni*. However, the isolates were not acidophilic (they grew well at pH 6.0–6.8), they did not perform malolactic fermentation and they did not grow in broth containing 10% (v/v) ethanol; these characteristics clearly distinguished the isolates from *O. oeni* (Table 1).

On the basis of 16S rRNA gene sequence analysis, the six isolates can be assigned to the genus *Oenococcus*, but the levels of DNA–DNA relatedness and the phenotypic characteristics indicated a clear separation of the isolates from the recognized species of the genus *Oenococcus*. Thus, the isolates represent a novel species, for which the name *Oenococcus kitaharae* sp. nov. is proposed. Furthermore, as some characteristics of *O. kitaharae* sp. nov. do not conform

to those of the genus *Oenococcus* (Dicks *et al.*, 1995), an emended description of the genus *Oenococcus* is also presented.

Emended description of *Oenococcus* Dicks *et al.* 1995

Oenococcus (Oe.no.coc'cus. Gr. n. *oinos* wine; N.L. masc. n. *coccus* from Gr. masc. n. *kokkus* berry; N.L. masc. n. *Oenococcus coccus* from wine).

The description of the genus *Oenococcus* is as given in detail by Dicks *et al.* (1995) with the following changes. Acidophilia, malolactic fermentation, growth in broth containing 10% (v/v) ethanol and growth stimulation by substances in tomato juice are dependent on species. The DNA G+C content ranges from 37 to 43 mol%. The type species is *Oenococcus oeni*.

Description of *Oenococcus kitaharae* sp. nov.

Oenococcus kitaharae (ki.ta.ha'rae. N.L. gen. n. *kitaharae* of Kitahara, to honour the Japanese microbiologist Kakuo Kitahara, for his contribution to the systematics and classification of lactic acid bacteria and in the development of the study of lactic acid bacteria).

Cells are Gram-positive, non-motile and small ellipsoidal cocci measuring 0.2–0.4 by 0.5–0.8 µm. Cells usually occur in pairs. Facultatively anaerobic and catalase-negative. Growth on agar medium is enhanced under anaerobic conditions. Colonies are white, smooth and approximately 1 mm in diameter on MRS/BHI agar and less than 1 mm in diameter on MRS agar under aerobic conditions after incubation for 7 days at 30 °C. However, on both media, colonies grown under anaerobic conditions are approximately 2 mm in diameter after incubation for 5 days at 30 °C. They are heterofermentative and produce lactic acid, carbon dioxide and ethanol or acetic acid from D-glucose. D-Lactate and L-lactate are produced in the ratio 9:1. Nitrate is not reduced. Acid is produced from D-glucose, D-fructose, D-galactose, D-mannose, maltose, melibiose and D-trehalose and produced weakly from D-ribose, D-glucosate and raffinose, but not from L-arabinose, D-xylose, L-rhamnose, lactose, sucrose, D-melezitose, D-mannitol, D-sorbitol or starch. Acid production from cellobiose and

Table 1. Differential characteristics of strain NRIC 0645^T and *O. oeni*

Data for *O. oeni* strains are from Garvie (1967) and Dicks *et al.* (1995).

Characteristic	Strain NRIC 0645 ^T	<i>O. oeni</i>
Growth in broth containing 10% ethanol	–	+
Malolactic fermentation	–	+
Optimum pH for growth	6.0–6.8	4.8
Optimum temperature for growth	30	22
Acid from maltose	+	–

D-salicin is strain-dependent. Dextran is not formed from sucrose. L-Malate is not decarboxylated to L-lactate in the presence of a fermentable carbohydrate. Cells grow between 20 and 30 °C but not at 15 or 37 °C. The optimum temperature for growth is 30 °C. All strains grow at pH values ranging from 5.0 to 7.5; some strains grow at pH 4.5. The optimum pH for growth is between 6.0 and 6.8. Growth is not stimulated in the presence of tomato juice. Growth is observed in MRS/BHI broth containing 1 % (w/v) NaCl but not 2.5 % (w/v) NaCl. Growth is also observed in broth containing 5 % (v/v) ethanol but not 10 % (v/v) ethanol. The DNA G + C content ranges from 41 to 43 mol% (type strain, 41 mol%).

The type strain is NRIC 0645^T (=JCM 13282^T=DSM 17330^T). All known strains were isolated in 2003 from a composting distilled shochu residue collected at a shochu distillery in Miyazaki Prefecture in the southern Kyushu area of Japan.

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