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

Akihito Endo and Sanae Okada

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 0645T (= JCM 13282T = DSM 17330T).

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 0645T, 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 0331T 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 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 method (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 0645T 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 0645T was used to search for sequence similarity with DataBase, and approximately 1450 bp of the 16S rRNA gene sequences of strain NRIC 0645T and related species were used to construct a phylogenetic tree. The highest level of sequence similarity to strain NRIC 0645T was found with O. oeni (96.0 %). In particular, the similarity of a partial sequence of strain NRIC 0645T [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 0645T 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. 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 0331T. 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 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 method (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 0645T 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 0645T was used to search for sequence similarity with DataBase, and approximately 1450 bp of the 16S rRNA gene sequences of strain NRIC 0645T and related species were used to construct a phylogenetic tree. The highest level of sequence similarity to strain NRIC 0645T was found with O. oeni (96.0 %). In particular, the similarity of a partial sequence of strain NRIC 0645T [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 0645T 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 0645T (positions 8–531). Strain NRIC 0645T 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 0331T 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 0331T. 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 0645T and related taxa on the basis of 16S rRNA gene sequences. Lactococcus lactis ATCC 19435T was used as an outgroup. Bootstrap percentages above 70 % are given at branching points.
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 6–6.8), 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’us. 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-glucurate 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).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain NRIC 0645^T^</th>
<th><em>O. oeni</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth in broth containing 10% ethanol</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Malolactic fermentation</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Optimum pH for growth</td>
<td>6–0–6.8</td>
<td>4–8</td>
</tr>
<tr>
<td>Optimum temperature for growth</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Acid from maltose</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>
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.

Acknowledgements

We thank the owner and staff of Akashi Distillery Ltd, Miyazaki, Japan, for providing the fermentation samples. We also thank K. Komagata for valuable discussions and N. Tanaka (Center for Information Biology and DNA Database of Japan, National Institute of Genetics, Shizuoka) for advice regarding the construction of phylogenetic trees using maximum parsimony and maximum likelihood. We are also grateful to R. Tsuji and J. Iida (NODAI Culture Collection Center, Faculty of Applied Bioscience, Tokyo University of Agriculture) for their technical assistance.

References


