Acetobacter oeni sp. nov., isolated from spoiled red wine

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A bacterial strain, designated B13^T, was isolated from spoiled red wine from the Dão region, Portugal. The strain was Gram-negative, strictly aerobic, rod-shaped and motile. Phylogenetic analysis on the basis of 16S rRNA gene sequences indicated that B13^T belonged to the genus *Acetobacter* within the *Alphaproteobacteria*. The closest related species was *Acetobacter aceti*, with 98.4% 16S rRNA gene sequence similarity. DNA–DNA hybridization showed that B13^T constituted a taxon separate from the *Acetobacter* species with validly published names. The DNA G+C content of B13^T was 58.1 mol%. Phenotypic characteristics of B13^T allowed its differentiation from the recognized *Acetobacter* species. B13^T produced 5-ketogluconic acid from glucose, but no 2-ketogluconic acid. It produced catalase, but no oxidase. It utilized glycerol, but not maltose, ethanol or methanol as carbon sources. On the basis of the results obtained, B13^T represents a novel species for which the name *Acetobacter oeni* sp. nov. is proposed. The type strain is B13^T (=LMG 21952^T = CECT 5830^T).

The genus *Acetobacter* currently comprises 23 species with validly published names, nine of which have been subsequently transferred to other genera. The *Acetobacter* species were delineated mainly on the basis of DNA–DNA relatedness and phylogenetic relationships (Sokollek *et al.*, 1998; Lisdiyanti *et al.*, 2000, 2001; Cleenwerck *et al.*, 2002).

During a study of red wine from the Dão region, Portugal, that had been spoiled due to the presence of volatile phenols produced by *Dekkera bruxellensis* (Silva *et al.*, 2004), a bacterial strain, B13^T, was recovered. The strain was isolated by spreading 1 ml spoiled red wine samples aseptically on YEDC plates (1⁻¹: 7 g glucose, 3 g yeast extract, 20 g calcium carbonate and 17 g agar) where it grew well and produced large haloes due to the solubilization of calcium carbonate by the large amounts of acid secreted by the strain. On this agar, colonies of the novel isolate were mucoid and opaque cream.

Strain B13^T was grown on nutrient agar for 48 h at 22 °C to check for motility by phase-contrast microscopy. Gram staining of cells was carried out according to the classical

Gram procedure described by Doetsch (1981). Strain B13^T was a Gram-negative, rod-shaped and motile organism with peritrichous flagella (0.8–0.9 × 3.6–5.1 µm).

DNA for 16S rRNA gene sequencing was extracted as described by Rivas *et al.* (2001). Amplification and sequencing of the nearly complete 16S rRNA gene were performed as described previously (Rivas *et al.*, 2003). The sequence determined was compared with sequences from GenBank using the BLAST program (Altschul *et al.*, 1990). Phylogenetic analysis was performed using the BIONUMERICS 4.0 software package (Applied Maths). The sequence determined was aligned with similar sequences retrieved from the EMBL database. Nucleotide substitution rates were calculated using the two-parameter method of Kimura (1980). The phylogenetic tree was inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis based on 1000 replications was undertaken to test the robustness of the phylogenetic tree (Felsenstein, 1985).

The nearly complete 16S rRNA gene sequence of B13^T (1484 nucleotides) was obtained. B13^T was located in the *Alphaproteobacteria*, within the genus *Acetobacter*. The sequence similarities of B13^T to the type strains of *Acetobacter aceti*, *Acetobacter tropicalis*, *Acetobacter estunensis*, *Acetobacter cerevisiae*, *Acetobacter indonesiensis*, *Acetobacter*

Published online ahead of print on 26 August 2005 as DOI 10.1099/ij.s.0.46000-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Acetobacter oeni* sp. nov. B13^T is AY829472.

malorum, *Acetobacter orleanensis*, *Acetobacter orientalis*, *Acetobacter cibinongensis*, *Acetobacter syzygii*, *Acetobacter lovaniensis*, *Acetobacter pomorum*, *Acetobacter pasteurianus* and *Acetobacter peroxydans* were 98.3, 98.1, 98.0, 97.9, 97.9, 97.8, 97.6, 97.5, 97.4, 97.1, 97.0, 96.7 and 96.4% respectively. Fig. 1 shows the phylogenetic position of strain B13^T within the genus *Acetobacter*.

DNA for DNA–DNA hybridizations and DNA base composition analysis was prepared by the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002). DNA–DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989) (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002). The hybridization temperature was 46 °C. Reciprocal reactions (e.g. A × B and B × A) were performed and the variation between them was within the limits of this method (Goris *et al.*, 1998). The DNA–DNA relatedness percentages presented are the means of a minimum of four hybridization experiments, including the reciprocal reactions. Strain B13^T showed low DNA–DNA relatedness ($\leq 20\%$) with the type strains of *A. aceti* (13%), *A. tropicalis* (14%), *A. estunensis* (20%), *A. cerevisiae* (13%), *A. indonesiensis* (7%), *A. malorum* (5%), *A. orleanensis* (6%), *A. orientalis* (6%), *A. cibinongensis* (7%), *A. syzygii* (10%), *A. lovaniensis* (15%), *A. pomorum* (12%), *A. pasteurianus* (16%) and *A. peroxydans* (13%). The DNA G + C content was determined by HPLC according to the method of Mesbah *et al.* (1989). Non-methylated phage lambda DNA (Sigma) was used as the calibration reference. The DNA G + C content of B13^T was 58.1 mol%.

Phenotypic characteristics were examined as described by Cleenwerck *et al.* (2002). The phenotypic characteristics of B13^T are given in the species description. Phenotypic characteristics that differentiate B13^T from recognized *Acetobacter* species are given in Table 1. Strain B13^T can be differentiated from the other species of the genus *Acetobacter* on the basis of 2- and/or 5-ketogluconic acid production from D-glucose, which are the main diagnostic characters for this genus. The ability of the strain to grow in the presence of 10% ethanol also distinguishes strain B13^T from most *Acetobacter* species. Moreover, strain B13^T differs from the closest phylogenetically related species, *A. aceti* and *A. estunensis*, as it is unable to grow with ammonium as the sole nitrogen source with ethanol as the carbon source.

The results presented above allow the genotypic and phenotypic differentiation of B13^T from the 14 *Acetobacter* species with validly published names. Strain B13^T should therefore be classified as representing a novel species for which we propose the name *Acetobacter oeni* sp. nov.

Description of *Acetobacter oeni* sp. nov.

Acetobacter oeni (oe'ni. N.L. gen. n. *oeni* of wine from Gr. gen. n. *oinou* of wine).

Cells are motile, non-spore-forming rods, 3.6–5.1 µm in length and 0.8–0.9 µm in diameter. Gram-negative, strictly aerobic. The optimal growth temperature on YEDC is 28 °C. Colonies on YEDC are circular, convex, cream, opaque and usually 1–2 mm in diameter within 4 days growth at 28 °C.

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Acetobacter oeni* B13^T and other related species of the family *Acetobacteraceae*. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotides.

Table 1. Differential characteristics of *Acetobacter oeni* B13^T compared with closely related species

Taxa: 1, *A. cerevisiae* (four strains); 2, *A. malorum* LMG 1746^T; 3, *A. pasteurianus* (seven strains); 4, *A. pomorum* LMG 18848^T; 5, *A. peroxydans* (two strains); 6, *A. lovaniensis* LMG 1617^T; 7, *A. orleanensis* (four strains); 8, *A. indonesiensis* (two strains); 9, *A. tropicalis* (two strains); 10, *A. estunensis* (three strains); 11, *A. acetii* (four strains); 12, *A. syzygii* LMG 21419^T; 13, *A. cibinongensis* LMG 21418^T; 14, *A. orientalis* LMG 21417^T; 15, *A. oeni* B13^T. +, Positive; -, negative; w, weakly positive; v, variable; ND, not determined. Data for taxa 1–11 were taken from Cleenwerck *et al.* (2002); data for taxa 12–15 were obtained in this study.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Formation from D-glucose:															
5-Keto-D-gluconic acid	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
2-Keto-D-gluconic acid	+	+	v	-	-	+	+	+	+	+	+	-	+	+	-
Growth in ammonium with ethanol	-	-	-	-	+	+	-	-	-	+	+	-	w	-	-
Growth in presence of 10% ethanol	ND	ND	+	*	*	*	*	*	*	*	*	*	*	*	+
Growth on carbon sources:															
Glycerol	+	+	v	+	-	+	+	+	+	v	+	+	+	+	+
Maltose	-	-	v	-	+	-	v	+	+	-	v	+	-	w	-
Methanol	-	+	-	-	-	+	-	-	-	-	-	-	-	w	-
Growth on YE + 30% D-glucose	-	+	v	-	-	-	-	-	-	-	-	-	+	-	-
Catalase	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
DNA G+C content (mol%)	56.0–57.6	57.2	53.2–54.3	52.1	59.7–60.7	57.1–58.9	55.7–58.1	54.0–54.2	55.6–56.2	59.2–60.2	56.9–58.3	54.3–55.4	53.8–54.5	52.0–52.8	58.1

*Data taken from Lisdiyanti *et al.* (2001).

Produces 5-keto-D-gluconic acid from D-glucose but no 2-keto-D-gluconic acid. Oxidizes D-glucose in media containing ammonium nitrate as the nitrogen source, but is unable to ferment D-glucose in the same media. Produces catalase, but no oxidase. Unable to grow with ammonium as the nitrogen source with ethanol as carbon source. Growth in presence of 10% ethanol. Utilizes glycerol as a carbon source, but not maltose or methanol. No growth in the presence of 30% D-glucose. DNA G+C content of 58.1 mol%.

The type strain, B13^T (=LMG 21952^T=CECT 5830^T), was isolated from spoiled red wine of the Dão region, Portugal.

Acknowledgements

This work was supported by CAICYT-DGES and JCyL (Spanish Government). We wish to thank K. Engelbeen for her technical assistance with the DNA–DNA hybridizations and phenotypic tests. A. W. is grateful to the Fund for Scientific Research – Flanders for a postdoctoral fellowship.

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