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Leuconostoc fallax, an Acid and Ethanol Tolerant Lactic Acid Bacterium

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Abstract: *Leuconostoc fallax*, known to be present in sauerkraut, was reisolated from exudates of *Gerbera jamesonii*. The identity of the isolates with *L fallax* was demonstrated by sequence analysis of the first 600 bases of the 16S rRNA. *L fallax* utilised a small number of sugars and showed a remarkable resistance to lactic acid. The final pH in glucose broth was 3·9. Moreover it was able to grow in the presence of ethanol (9·0% v/v) and salt (5·5%), but it was unable to carry out a malo-lactic fermentation.

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INTRODUCTION

From exudates of *Gerbera* plants a lactic acid bacterium was isolated. Many cultivars of *G jamesonii* are grown in greenhouses. After cutting the flowers, some slimy liquid flows out of the stalks. After some time this exudate becomes white due to the growth of the yeast-like fungus *Galactomyces geotrichum* (Butler et Petersen) Redhead et Malloch (syn *Geotrichum candidum* Link) (Van Kesteren *et al* 1992). Sometimes the stalks and rosettes rot and a characteristic sweet odour is exhaled. This plant disease, colloquially called 'sugar rot', often causes death. The microbial agent causing it is still unknown. In an attempt to isolate this phytopathogenic microorganism, the microflora of the slimy efflux was analysed. In addition to *G geotrichum* and a coryneform bacterium with yellow colonies, many small catalase-negative colonies appeared. The present paper describes some properties of this lactic acid bacterium and presents evidence for its identity with *Leuconostoc fallax* Martinez-Murcia and Collins (1991).

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MATERIALS AND METHODS

Isolation of the strains

The slimy exudate flowing from the stalks after cutting the flowers of *G jamesonii* was streaked on YM agar (10 g glucose, 5 g yeast extract, 3 g peptone, 3 g malt extract, 20 g agar, pH 6·5, per litre of demineralised water). After 2–3 days incubation at 25°C many small catalase-negative colonies appeared between yeast colonies of *G geotrichum* and yellow colonies of a coryneform bacterium. Microscopic examination of these small colonies revealed the presence of Gram-positive streptococci, presumably lactic acid bacteria. After streaking on YM agar, the bacterium was maintained in

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stab cultures of YM agar. Two strains were studied in detail: Ge-1 was isolated from slimy exudate about 1 day after cutting the flower; Ge-2 was isolated from exudate on which growth of *G geotrichum* was apparent. Both strains were deposited in the culture collection of the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, Germany.

Analysis of culture liquids

In some cultures the fermentation products were analysed by high-performance liquid chromatography (HPLC) using a Chrompack Organic Acid Column (Chrompack, Middelburg, The Netherlands) provided with a Micro Guard Carbo P head column and an infrared detector. The eluant (0·60 ml min^{-1}) was 10 mM sulphuric acid. This HPLC method allowed accurate measurements of lactic and acetic acids and of ethanol.

Physiological studies

The pattern of sugar fermentation was studied using the API 50 CH identification kit (bioMérieux, Marcy-l'Etoile, France). The results were confirmed by a growth test in a medium consisting of yeast extract (10 g l^{-1}), peptone (10 g l^{-1}) and the sugar under investigation (10 g l^{-1}). The initial pH was 6·5. Amounts of 2·5 ml of these media were inoculated with a droplet of a preculture (24 h, 37°C) in glucose broth. Incubation took place at 25°C for up to 3 weeks. Utilisation of L-malic and citric acids, partially neutralised with sodium hydroxide, was studied in the same medium; the initial pH was 5·0. Resistance to ethanol and to sodium chloride at various concentrations of these inhibitors was tested in glucose (20 g l^{-1}), yeast extract (10 g l^{-1}), peptone broth (10 g l^{-1}). These cultures (7·5 ml in culture tubes) were supplied with 1 ml sterile liquid paraffin in order to prevent evaporation and were incubated at 25°C. Sensitivity to vancomycin and ampicillin at 100 mg l^{-1} was tested in the same medium.

Analysis of 16S rRNA

For the genetic characterization of the strains, DNA was isolated from one colony using the BioRad (Veenendaal, The Netherlands) matrix according to the supplier's protocol. These DNA extracts were used directly for partial amplification of the 16S rRNA and the analysis by RAPD and REP patterns. Part of the 16S rRNA was amplified by using the following PCR primers based on conserved sequences: P1 [GCGGCGTGCCTAATACATGC] (positions 41 to 60 in the *E coli* numbering system) and P2 [ATCTACGCATTTCACCGCTAC] (complementary to positions 685–705 in the *E coli* numbering system). The same primers were subsequently used in the direct sequencing of both strands of the amplified DNA. The PCR amplifications were performed by using a Perkin Elmer Thermocycler 480 (Perkin Elmer, Nieuwerkerk aan den IJssel, The Netherlands). The reactions were carried out in sterile 0·5-ml tubes, which contained 45 µl of the following buffer: 10 mM Tris-HCl (pH 8·8), 1·5 mM $MgCl_2$, 50 mM NaCl, deoxynucleotide triphosphates at 2·5 mM, 100 ng of each primer and 1 unit of Taq polymerase (Pharmacia, Roozendaal, The Netherlands). Template DNA (5 µl) was added after being heated to 95°C to eliminate all protease activity. The amplification was done in 30 cycles by melting the DNA at 93°C for 1 min, annealing at 54°C for 1·5 min and elongation at 72°C for 2·5 min. After PCR amplification the DNA was purified by EasyPrep™ (Pharmacia, Roozendaal, The Netherlands) according to the protocol of the manufacturer. The amplified DNA was sequenced using the thermosequenase kit (Amersham, 's-Hertogenbosch, The Netherlands) and the ALF automated sequencer (Pharmacia, Roozendaal, The Netherlands). Both strands were sequenced using fluorescent-labelled primers (P1 and P2). The 16S rRNA sequence obtained was compared to 16S rRNA sequences in the RPD database (Madiak *et al* 1994). The received identification was checked manually.

RESULTS

Physiology and identification

Two strains of the lactic acid bacterium were studied in detail: viz. Ge-1 and Ge-2. Gas was produced during the fermentation of glucose broth. Both strains grew as ovoid cocci in long twisted chains of up to 40 cells. These properties, as well as their aerotolerance, indicate that both strains belong to the genus *Leuconostoc* van Tieghem. Some other properties are in agreement with this diagnosis: both strains are resistant to vancomycin like most *Leuconostoc* species (Orberg *et al* 1984) and sensitive to ampicillin. The arginine deaminase reaction is negative (Garvie 1986).

The final pH in glucose (20 g l^{-1}) broth was 3·9. In a glucose broth with yeast extract and peptone each at 5 g l^{-1} the final pH was as low as 3·5. Most *Leuconostoc* species, *L oenos* Garvie excepted, fail to grow at pH 4·8 (Garvie 1986). Both isolates from *Gerbera* grew in the presence of 9% (v/v) ethanol, another property characteristic of *L oenos*. However, this bacterium is slow growing and fastidious (Garvie 1967, 1986).

Identity of both *Gerbera* strains with *L fallax* Martinez-Murcia and Collins 1991 was presumed, as both strains responded similarly to the type strain (DSM 20189) of this species in the API 50 CHL identification system. However, acid production from mannitol and trehalose occurred within 2 and 4 days, respectively, and was not delayed like in the type strain (Martinez-Murcia and Collins 1991). Growth responses

of the three strains in yeast extract peptone broth are listed in Table 1. Again, growth on mannitol of strains Ge-1 and Ge-2 was more rapid than that of the type strain, and the latter failed to grow on ribose and trehalose. The type strain was originally described as large cocci occurring singly or in pairs (Martinez-Murcia and Collins 1991). These differences in phenotypical properties threw some doubt on the identification of the strains as *L fallax*. In order to put this beyond doubt, sequence analyses of the 16S rRNA of strains Ge-1 and Ge-2 were carried out and were compared with the data published by Martinez-Murcia and Collins (1991). Sequence analysis of the first 600 bases revealed that strains Ge-1 and Ge-2 have the same 16S rRNA as *L fallax*. This confirmed their identification as *L fallax*.

TABLE 1
Utilization of carbon compounds by three strains of *Leuconostoc fallax*[a]

	Strain		
	DSM 20189	*Ge-1*	*Ge-2*
Carbon compound			
D-Glucose	3 +	3 +	3 +
D-Fructose	3 +	3 +	3 +
D-Mannose	2 +	2 +	2 +
D-Galactose	—	—	—
L-Sorbose	—	—	—
D-Ribose	—	2 +	2 +
D-Xylose	—	—	—
D-Arabinose	—	—	—
L-Rhamnose	—	—	—
Sucrose	3 +	3 +	3 +
Maltose	—	D	D
Methyl-α-D-glucoside	3 +	3 +	3 +
Trehalose	—	2 +	2 +
Cellobiose	—	—	—
Melibiose	—	—	—
Lactose	—	—	—
Raffinose	—	—	—
Melezitose	—	—	—
Starch	—	—	—
Inulin	—	—	—
Glycerol	—	—	—
Erythritol	—	—	—
Adonitol	—	—	—
Xylitol	—	—	—
L-Arabinitol	—	—	—
Sorbitol	—	—	—
Mannitol	+	3 +	3 +
Galactitol	—	—	—
myo-Inositol	—	—	—
D-Glucosamine	3 +	3 +	3 +
Acetylglucosamine	3 +	3 +	3 +
D-Gluconate	3 +	3 +	3 +

[a] 3+ means dense growth within 1 day; 2+ means 2–4 days required, + 4–7 days, D longer period required for growth.

Further characterisation

The type strain of *L fallax*, DSM 20189, showed the same morphological characteristics as the strains from *Gerbera* exudate, ie long twisted chains of ovoid cocci. Growth singly or in pairs (Martinez-Murcia and Collins 1991) was not observed in the present study.

The final pH of standing cultures of strains DSM 20189, Ge-1 and Ge-2 (ie 10 ml 50 mM glucose broth pH 6·5 in culture tubes) was 3·86, 3·90 and 3·86, respectively. Weak growth occurred in similar cultures with an initial pH of 4·8; the final pH was also 3·9. This demonstrates that the type strain is as acid tolerant as strains Ge-1 and Ge-2. In aerobic cultures of both strains from *Gerbera* (ie 10 ml 50 mM glucose broth in a shaken Erlenmeyer flask of 100 ml), growth was slower and the final pH was 4·1. Strain DSM 20189 did not grow under these conditions. Analysis of the fermentation products revealed that in standing cultures about equimolar amounts (50 mM) of lactic acid and ethanol were formed, with traces of acetic acid. In the aerobic cultures of strains Ge-1 and Ge-2, no ethanol was detected. Equimolar amounts (20 mM) of lactic and acetic acids were produced instead.

All three strains of *L fallax* failed to grow in yeast extract peptone broth supplied with L-malic acid or citric acid of which the pH had been adjusted to 5·0 with sodium hydroxide. Addition of glucose (2 g l^{-1}) to these media allowed weak growth, to a final pH of 4·8. The Voges-Proskauer reaction in the citrate broth was negative.

All three strains grow at 4 and 40°C but not at 42°C. Abundant amounts of slime are produced on sucrose (20 g l^{-1}) yeast extract (10 g l^{-1}) peptone (10 g l^{-1}) agar (15 g l^{-1}). Standing cultures in glucose (20 g l^{-1}) broth supplied with different concentrations of ethanol and salt showed a remarkable tolerance to these inhibitors. After 5 days, all three strains grew in the presence of 9% (v/v) ethanol; after 10 days, strain Ge-1 showed weak growth with 10% ethanol. Sodium chloride at 5·5% (w/v) was tolerated by all strains. The type strain showed delayed growth (after 12 days) at 6·5%.

DISCUSSION

From *Gerbera* exudate three microorganisms were isolated: *G geotrichum*, a coryneform bacterium with yellow colonies and a lactic acid bacterium, identified as *L fallax* Martinez-Garcia et Collins. Inoculation of healthy *Gerbera* plants with these three microorganisms, either singly or combined, did not affect the plants. So they are probably not the cause of the 'sugar rot' disease.

Initially, identity of both isolates from *Gerbera* with *L oenos* was assumed, as both strains were acid and ethanol tolerant. However, this bacterium is slow-

growing and fastidious. It is only isolated from wine and related habitats where it carries out a malo-lactic fermentation (Garvie 1967). Glucopantothenate (tomato juice factor) is required for growth. Moreover, it prefers a low pH and grows very slowly at pH 6·5 (Garvie 1967, 1986). The isolates from *Gerbera* exudate grow very well in a glucose yeast extract peptone broth at pH 6·5 and prefer this medium to MRS medium, which is recommended for cultivation of lactic acid bacteria. Moreover, both isolates from *Gerbera* exudate failed to carry out the malo-lactic fermentation, as no growth occurred in malate broth. In this respect *L fallax* differs from many other *Leuconostoc* species (Garvie 1986). *L oenos* included (Garvie 1967).

Identity of strains Ge-1 and Ge-2 with *L fallax* was concluded from homology of the 16S rRNA and from great correspondence of the sugars fermented by the three strains involved. However, the growth response of both strains from *Gerbera* exudate differed somewhat from that of the type strain, DSM 20189 (Table 1). The latter had been described as large cocci growing singly or in pairs (Martinez-Garcia and Collins 1991). However, in the present study, strain DSM 20189 showed the same morphology as both isolates from *Gerbera* exudate, ie long twisted chains of ovoid cocci.

L fallax shares some properties with other species of this genus: heterofermentative catabolism of glucose, which under anaerobic conditions is completely converted into lactic acid , ethanol and carbon dioxide and aerobically partially into lactic and acetic acids. The incomplete conversion of glucose into its fermentation products under aerobic conditions may be due to toxicity of acetic acid or to production of hydrogen peroxide or other metabolites of oxygen. *L fallax* is distinguished from most other species in this genus by its tolerance to lactic acid and ethanol and by the absence of the malo-lactic fermentation. Growth in citrate broth and production of diacetyl, acetoin or butane-2,3-diol from citrate were also absent. Application of *L fallax* in the food industry is feasible and assumed to be safe, as the type strain originated from sauerkraut (Martinez-Garcia and Collins 1991). In particular its tolerance to ethanol, acid and salt make *L fallax* an attractive organism.

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