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NOTE

Isolation and identification of species of *Alicyclobacillus* from orchard soil in the Western Cape, South Africa

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Abstract Alicyclobacilli were isolated from orchard soil collected from an apple and pear farm in Elgin, Western Cape, South Africa. Morphological, biochemical and physiological characteristics of the isolates were used to presumptively classify them as belonging to the genus *Alicyclobacillus*. Strains were identified to species level by polymerase chain reaction (PCR) with genus-specific primers, and 16S ribosomal RNA (rRNA) gene sequencing. To our knowledge this is the first report on the isolation of *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius* from orchard soil. The presence of these organisms in the soil suggests a possible source of contamination for the final fruit juice, concentrate or pulp.

Keywords *Alicyclobacillus* · Soil · Orchard · *16S rRNA* gene

Introduction

The alicyclobacilli are thermo-acidophilic, Gram-positive, rod-shaped, spore-forming, aerobic micro-organisms that possess unique fatty acids (ω-cyclohexane or ω-cycloheptane) as the major components of the cellular membrane (Wisotzkey et al. 1992; Walls and Chuyate 1998). Species of *Alicyclobacillus* have been isolated from a range of habitats and substrates, such as organic compost, manure, fruit, and heat-processed foods (Deinhard et al. 1987; Yamazaki et al. 1996; Pettipher et al. 1997; Albuquerque et al. 2000; Jensen 2000; Walls and Chuyate 2000; Goto et al. 2002; Matsubara et al. 2002). At present, there are 12 recognized species of *Alicyclobacillus* of which three species have been isolated from spoilt juice products, namely, *Alicyclobacillus acidoterrestris*, *Alicyclobacillus acidocaldarius* and *Alicyclobacillus pomorum* (Goto et al. 2003; Jensen and Whitfield 2003; Gouws et al. 2005).

Alicyclobacillus spores are heat resistant, and pasteurization does not inactivate the spores (Splittstoesser et al. 1994; Eiroa et al. 1999; Orr and Beuchat 2000). This heat resistance was observed by Splittstoesser et al. (1994) who reported *D* values for *A. acidoterrestris* of 23 min at 90°C and 2.4–2.8 min at 95°C, suggesting that spores survive the typical juice pasteurization process that consists of holding at 86–96°C for 2 min. In fact, pasteurization serves as a heat treatment that stimulates germination of the spores, leading to growth. The ability of *A. acidoterrestris* to grow at a pH range of 2.5 to 6 (Yamazaki et al. 1996) and to survive the typical juice pasteurization process has caused concern in the fruit juice industry (Splittstoesser et al. 1998; Eiora et al. 1999; Gouws et al. 2005).

Spoilage caused by this micro-organism is visually difficult to detect. The spoiled juice appears normal or might have light sediment. No gas is produced. Often, the only evidence of spoilage is a medicinal/phenolic off-flavour (Walls and Chuyate 1998; Jensen 1999, 2000). Fruit juice contamination results from unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices (Pontius et al. 1998; Orr and Beuchat 2000; McIntyre et al. 1995). Our interest in the presence of species of

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Alicyclobacillus in orchard soil is to investigate a potential source of contamination of the final fruit juice concentrate. The aim of this study was to isolate potential spoilage causing species of *Alicyclobacillus* from orchard soil of an apple and pear farm. The isolated strains of *Alicyclobacillus* were identified to species level on the basis of genus-specific PCR and rRNA gene sequence analyses.

Materials and methods

Collection of samples

Top soil was collected from 12 random sites in apple and pear orchards on a farm in the Elgin region of the Western Cape, South Africa. The soil samples were sieved through a 2.0 mm width mesh to remove stones and plant debris. A soil:water ratio of 1:1 was used for the determination of soil pH. Approximately 10 g of soil was placed in sterile 50 ml centrifuge tubes and re-suspended in 30 ml sterile peptone water. After soil particles were allowed to sediment, the supernatants were heat treated at 80°C for 10 min (Walls and Chuyate 2000) and diluted in sterile distilled water. Dilution series of 10^{-1} to 10^{-6} were prepared in triplicate on potato dextrose agar (PDA) (Biolab, Biolab Diagnostics, Midrand, South Africa), orange serum agar (OSA) (Oxoid, Basingstoke, Hampshire, England), YSG (yeast extract starch glucose) agar (Uchino and Doi 1967) and YSG agar, supplemented with 20% (v/v) sterile apple juice. All media contained 100 μg ml^{-1} Delvocid (GistBrocades, Delf, The Netherlands) to inhibit the growth of yeast and fungi. Tartaric acid (1 N) was used to adjust all media to a final pH of 4. One of three plates was incubated at 55°C while the remaining two sets of plates were incubated at 45°C. Plates were incubated aerobically and examined for growth after 72, 96 and 120 h.

Preliminary identification of alicyclobacilli

Colonies were randomly selected from plates containing between 20 and 300 colonies and re-streaked on corresponding media to obtain pure cultures. All cultures were stored at –80°C in YSG broth adjusted to a pH of 4 with 1 N tartaric acid, supplemented with sterile glycerol (30%, v/v, final concentration). Gram reaction, oxidase and catalase activity were determined according to the methods described by Harrigan and McCance (1976). Gram-positive, oxidase positive, and catalase positive rods were selected for further examination.

Carbohydrate fermentations

Carbohydrate fermentation reactions were recorded by using the API 50 CHB system (BioMerieux, Marcy L'Etoile, France) according to the manufacturer's instructions. Incubation of all API strips was at 45°C, and results were recorded after 4 and 5 days.

Genus-specific PCR amplification

Isolates were grown in YSG broth adjusted to pH 4 for 4 days. Their genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA was amplified with primers CC16S-F (5′-CGT AGT TCG GAT TGC AGGG-3′) and CC16S-R (5′-GTG TTG CCG ACT CTC GTG-3′, Connor et al. 2005). PCR reactions were performed in a total volume of 25 μl containing 0.6 μM of each of the primers, 1.25 U *Taq* DNA polymerase (Promega), 1 × PCR reaction buffer containing $MgCl_2$, 1 μl of 99% (v/v) dimethyl sulphoxide (DMSO), 0.4 mM deoxyribonucleoside triphosphate (dNTPs) and 2 μl of the extracted DNA. PCR reactions were performed in the Eppendorf Mastercycler Personal. An initial 3 min denaturation at 95°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, and a final 2 min chain elongation at 72°C. *A. acidocaldarius* PMO1 and *A. acidoterrestris* SAO1 (Gouws et al. 2005) were used as reference strains.

DNA sequencing

The genomic DNA of isolates was amplified with primers F8 (5′-CAG GCA TCC AGA CTT TGA TYM TGG CTC AG-3′) and R1512 (5′-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3′), as used by Felske et al. (1997). PCR was according to the method described by Garbers et al. (2004). The amplified fragments of approximately 1.5 kb in size were purified using the High Pure PCR Purification Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The fragments were sequenced using the ABI PRISM 377 DNA Sequencer (PerkinElmer) at the DNA Sequencing Facility, Stellenbosch University and compared to sequences in GenBank using the BLAST search option.

Results and discussion

The soil pH ranged from 5.91 to 6.71 (Table 1), which is typical of orchard soil. Of the 12 random sites where soil

samples were taken, 5 tested positive for the presence of species of *Alicyclobacillus*. YSG plates incubated at 45°C contained the highest amount of microbial cells. From a total of 28 isolates, 5 were selected based on Gram reaction, morphology and catalase activity. All isolates were Gram-positive, rod-shaped, oxidase and catalase positive. Carbohydrate fermentation reactions recorded for 4 of the 5 isolates corresponded most closely to that of the type strain of *A. acidoterrestris* (Table 2). Variations in the fermentation of D-raffinose, starch, trehalose, D-turanose and xylitol were recorded (Table 2). Similar results have been reported for other strains of *A. acidoterrestris* (Chang and Kang 2004; Silva and Gibbs 2001; Goto et al. 2001). None of the strains fermented ribose and aditinol, which is characteristic for the type strain of *A. acidoterrestris* ATCC 49025^T (Goto et al. 2001).

DNA amplification with genus-specific primers on all four isolates produced a 134 bp fragment (data not shown), which was identical in size to that reported for *A. acidoterrestris* ATCC 49025^T, *A. cycloheptanicus* ATCC 49029 and *A. acidocaldarius* ATCC 43030 (Connor et al. 2005). Sequencing of the 16S rRNA gene amplicons for the five isolates of *Alicyclobacillus* and comparison with nucleotide sequences in GenBank, revealed DNA homology between 96.8 and 98.7% to *A. acidoterrestris* ATCC 49025^T (Table 3). Differences in DNA sequence data suggested that the strains of *A. acidoterrestris*, isolated from orchard soil do not represent a homogeneous collection.

The carbohydrate fermentation pattern of isolate ESO3 corresponded most closely to *A. acidocaldarius* ATCC 2700^T (Table 3). Erythritol was unable to be assimilated, which is a characteristic of *A. acidocaldarius* (Goto et al. 2001). PCR with genus-specific primers yielded a DNA fragment of 134 bp (data not shown); indicating isolate ESO3 belonged to the genus *Alicyclobacillus* (Connor et al. 2005). Sequence analysis of isolate ESO3 revealed 98.6%

Table 1 Origin of isolates

Isolate	Location	Soil type	Soil pH
ESO6	Granny Smith orchard	Sandy clay loam	6.69
ESO7	Golden Delicious orchard	Sandy clay loam	6.50
ESO14	Forelle orchard	Sandy clay loam	6.66
ESO12	Fuji orchard	Sandy clay loam	6.71
ESO3	Packham's Triumph orchard	Clay loam	5.91

Table 2 Differential carbohydrate fermentation reactions of *Alicyclobacillus* isolates collected from orchard soil in the Western Cape, South Africa

Isolate	Glycerol	Erythritol	Ribose	Adonitol	L-Sorbose	Rhamnose	Trehalose	Inositol	Sorbitol	α-Methyl-D-mannoside	Amygdalin
A. acidoterrestris ATCC 49025Ta	+	+	+	+	–	+	–	+	+	+	+
ESO6	+	+	–	–	–	+	+	d	+	+	+
ESO7	+	+	–	–	–	+	+	d	+	+	+
ESO12	+	+	–	–	–	+	–	d	+	+	+
ESO14	+	+	–	–	–	+	–	d	+	+	+
A. acidocaldarius ATCC 27009Ta	+	–	+	–	+	+	–	–	–	–	–
ESO3	+	–	–	–	+	–	–	–	–	–	d

Isolate	Salicin	Melibiose	Melezitose	D-Raffinose	Starch	Glycogen	Xylitol	β-Gentiobiose	D-Turanose	D-Arabitol
A. acidoterrestris ATCC 49025Ta	+	–	+	–	–	–	+	+	+	+
ESO6	+	–	+	+	+	–	+	+	d	+
ESO7	+	–	+	+	d	–	d	+	d	+
ESO12	+	–	+	–	–	–	–	+	+	+
ESO14	+	–	+	–	–	–	–	+	–	+
A. acidocaldarius ATCC 27009Ta	–	+	–	+	–	+	–	–	+	+
ESO3	+	+	+	d	–	–	–	+	+	–

+, positive reaction; –, negative reaction; d, variable reaction. All strains fermented: aesculin, L-arabinose, arbutin, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, mannitol, D-mannose, α-methyl-D-glucoside, sucrose, trehalose and D-xylose. None of the strains fermented: *N*-acetyl-glucosamine, D-arabinose, L-arabitol, dulcitol, D-fucose, L-fucose, gluconate, inulin, 2-keto-gluconate, 5-keto-gluconate, β-methyl-xyloside, D-tagatose, D-xylose and L-xylose

[a] Data from Goto et al. (2001)

Table 3 Percentage similarity of isolates from orchard soil to species in the NCBI nucleotide sequence database on the basis of partial 16S rDNA sequence analysis

Isolate	No of nucleotides	Similarity (%)	Nearest phylogenetic neighbour (GenBank accession number)
ESO6	833	98.7	*Alicyclobacillus acidoterrestris* (AY686617.1)
ESO7	840	98.1	*Alicyclobacillus acidoterrestris* (AY686617.1)
ESO14	857	97.0	*Alicyclobacillus acidoterrestris* (AJ133631.1)
ESO12	845	96.8	*Alicyclobacillus acidoterrestris* (ABO42058.1)
ESO3	823	98.6	*Alicyclobacillus acidocaldarius* (ABO59665.1)

homology to the 16S rDNA of *A. acidocaldarius* DSM 451 (Table 3). Isolate ESO3 is thus regarded as a member of *A. acidocaldarius*.

To our knowledge, this is the first report on the isolation of *A. acidoterrestris* and *A. acidocaldarius* from orchard soil. Soil adhering to fruit bins, machinery and on fruit that was picked off the ground during harvest can greatly complicate subsequent cleaning operations in the processing plant. The presence of known spoilage causing species of *Alicyclobacillus* in orchard soil suggests a potential source of contamination for the final fruit juice, concentrate or pulp. Further research is needed to establish the role played by these specific strains in the spoilage of fruit juice.

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